SELECTIVE IgA DEFICIENCY IN CHILDHOOD

Clinical manifestations and in vitro experiments

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1 IMMUNOGLOBULINS

One of the activities of the immune system is the formation of antibodies. The proteins that exert antibody activity are called immunoglobulins. Immunoglobulin monomers have structural similarities as they all are composed of four polypeptide chains: two identical heavy chains and two identical light chains, linked together by inter- and intra-chain disulphide bridges. The ultra-structural configuration of an immunoglobulin is Y-shaped (Figure 1.1). Both heavy and light chains consist of several domains. The variable domains (V_H and V_L) are situated at the N-terminal and form the antigen binding site. The constant domains (C_H and C_L) are situated on the C-terminal of the polypeptide chain (1).

In humans five immunoglobulin classes named IgG, IgA, IgM, IgD and IgE can be distinguished. The distinction is made upon structural differences in the constant domains of the heavy polypeptide chains.

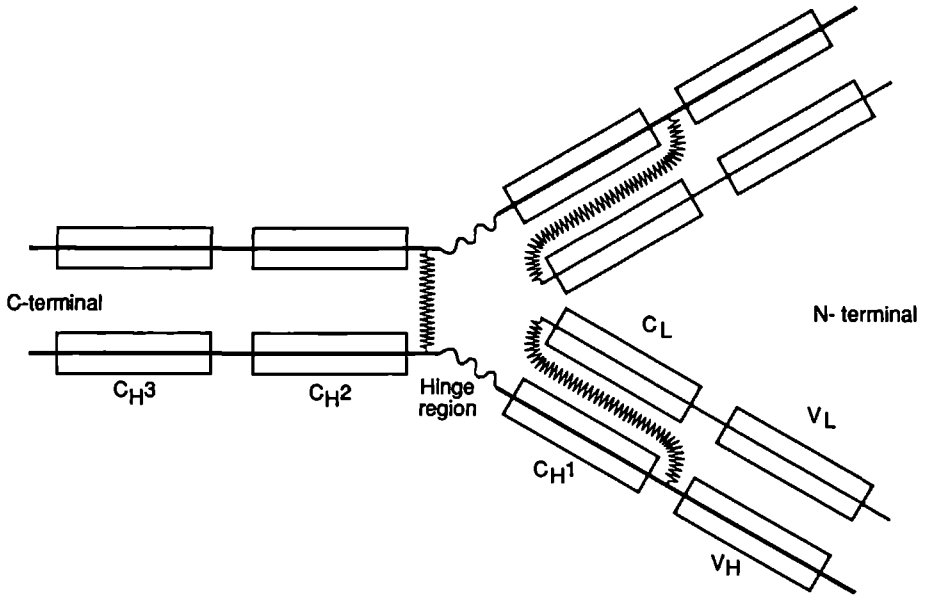

Figure 1.1: Schematic representation of a monomeric IgA molecule.

1.2 IMMUNOGLOBULIN A (IgA)

1.2.1 Structure, distribution and function of IgA

The IgA monomer consists of two heavy (alpha) polypeptide chains and two light chains (kappa or lambda). IgA is a minor component of serum immunoglobulin (approximately 10-20%), but it is the most prevalent immunoglobulin on mucosal surfaces and in secretory fluids such as human milk, saliva, tears and secretions of the urogenital, respiratory and gastro-intestinal tract. Because of the abundance of IgA-secreting plasma cells in submucosal lymphoid aggregates, the total number of IgA molecules produced in humans is greater than that of any other immunoglobulin class (2). It has been estimated, for comparison, that in an adult the synthetic rates (mg of Ig/kg body weight/day) are approximately 66 for IgA, 34 for IgG, 7.9 for IgM, 0.4 for IgD and 0.02 for IgE (3).

Two IgA subclasses named IgA_1 and IgA_2 can be recognized by small antigenic differences in the constant region of the alpha heavy chain. Differences between IgA_1 and IgA_2 in the hinge region (Figure 1.1) of the alpha chain lead to a different susceptibility to attack by proteolytic enzymes produced by several common bacterial species. Due to a deletion of a certain amino acid sequence in the hinge region of the alpha-2 chain, IgA_2 is resistant to these enzymes (2). Circulating IgA consists of 90% IgA_1 and 10% IgA_2, whereas the IgA subclasses are about equally distributed in secretions (1).

Serum IgA is mainly found in the monomeric form and is produced by plasma cells located predominantly in the bone marrow and the spleen. Although serum IgA has antibody activity against a variety of antigens, the exact function of serum IgA in the immunological defense system remains unclear. IgA does not fix complement in the classical pathway, and it has been suggested that the role of serum IgA is to bind foreign antigens to large immune complexes that can be removed by the phagocytic system without inflammatory phenomena due to complement activation (4).

The IgA found in secretions is largely dimeric, and is produced in plasma cells at local (submucosal) sites (1,2). Secretory IgA contains two extra chains: the J (joining or junction) chain and the secretory component (SC). The J chain is a 15,600 dalton polypeptide, produced by B lymphocytes, that binds two cysteine residues near the C-terminal of alpha chains and serves in the dimerization process of secretory IgA.

SC is a glycoprotein with, depending on the method of determination, a molecular weight of 50,000-90,000 dalton. It is produced in epithelial cells lining the mucosal surfaces (5-7). SC binds to IgA and serves in the transport of dimeric J chain-containing IgA into secretions. The resulting secretory IgA molecule is the predominant immunoglobulin in exocrine secretions and plays a primary role in mucosal immunity. Virtually all antigens presented to mucosal surfaces from the external environment stimulate the secretion of IgA antibodies. Secretory IgA forms complexes with antigens at the mucosal surfaces, thus preventing mucosal penetration of foreign antigens. The spectrum of activities is wide: it can neutralize viruses, bind toxins, agglutinate bacteria, inhibit bacteria from binding to cells, and bind to various food antigens (1,2,7).

1.2.2 Ontogeny of IgA

Compared to other immunoglobulins the development of IgA levels throughout life is slow. At birth, cord serum IgA concentrations have been estimated at 0.004 g/l (8). Unlike IgG, IgA cannot pass the placental barrier, so this IgA is likely to be of fetal origin. Serum IgA levels rise slowly during childhood, and adult values (1.4-2.6 g/l) are reached only at puberty (9). Maturation of the secretory IgA system is more rapid. Adult values of secretory IgA have been reported in saliva within two months after birth in an early study (10), but in a later study adult values for secretory IgA in stimulated saliva were attained at the age of 4-6 years (11). Distribution of plasma cells in the intestines is reported to be mature at the age of two years (12).

1.2.3 IgA and breast-feeding

Secretory IgA is also the predominant immunoglobulin in human milk. In fact, the observation that mother's milk contained an IgA that differed from serum IgA led to the first description of what was called to be secretory IgA (7). It has been recognized that breast milk antibodies were not absorbed, but functioned on the mucosal surfaces as a mucosal immunoglobulin in the gastro-intestinal tract of the infant.
Secretory IgA is relatively stable, and a large part of milk IgA can be recovered from stool. Secretory IgA concentration can be very high in colostrum, but it comes down to a stable level of 50-75 mg/dl after the

first few weeks (7). The amount of secretory IgA delivered to a fully breast-fed infant is estimated to be at least 0.5 gram daily (100 mg secretory IgA/kg/day). Intestinal and bronchial antigen exposure of mothers can result in the appearance of secretory IgA antibodies in breast milk and other secretions, suggesting an entero-mammaric as well as a broncho-mammaric pathway of lymphoid cells that, after being primed with antigen, are involved in the homing process and are destined to become local immunoglobulin-secreting plasma cells (7).

Numerous reports have been published stating the beneficial effects of breast feeding with its many defense factors (besides secretory IgA: lymphoid cells and other soluble factors as lactoferrin, enzymes and glycocompounds) upon prevention of infections and food allergy in the suckling infant.

1.3 SELECTIVE IgA DEFICIENCY

1.3.1 *Definition of selective IgA deficiency*

Deficiency of serum IgA was first described in 1961 by Thieffry et al. in patients with ataxia telangiectasia (13). True cases of selective IgA deficiency were first described in 1962 by West et al. (14).
Selective or isolated IgA deficiency was defined by Ammann and Hong in a restricted sense as a state with markedly diminished serum IgA levels (below 50 mg/l), normal levels of other immunoglobulins, normal antibody-mediated immunity and normal cell-mediated immunity (15). More recently the definition has been modified by not excluding a priori all patients with T cell abnormalities. Selective IgA deficiency now is defined as serum IgA levels below 50 mg/l, normal total levels of serum IgG and IgM, and no or at most mild abnormalities in T cell function (16). Patients with major immunodeficiency states as panhypogammaglobulinemia, or clearly defined syndromes as ataxia telangiectasia are thus excluded. Some authors have introduced the term "partial" IgA deficiency for individuals with serum IgA levels above 50 mg/l but below -2SD of age-specific reference values (17).

Serum IgA deficiency is almost invariably accompanied by deficiency of secretory IgA, although isolated deficiencies of either serum or secretory IgA have been described in some patients (16,18). Concomitant deficiencies of IgG subclasses, and/or IgD and/or IgE have been demonstrated in patients with "selective" IgA deficiency (19-23).

1.3.2 Frequency and genetics of IgA deficiency

Selective IgA deficiency is the most common primary immunodeficiency in humans. The occurrence of selective IgA deficiency has been studied extensively in healthy individuals (mostly blood donors), and in numerous patient groups. In blood donors the frequency of selective IgA deficiency varies from 1:300 to 1:18,500 depending on the population and the chosen lower IgA limit for IgA deficiency (24-26). In patient groups elevated frequencies have been reported many times (25). In a survey of 486 healthy dutch school children four cases of selective IgA deficiency were found (1:122) (9).

Most cases of IgA deficiency are sporadic, but familial occurrence has been reported. The patterns of inheritance are diverse. Autosomal dominant with or without variable expression, autosomal recessive, polygenic and multifactorial inheritance has been described (2,27-31). IgA heavy chain constant region genes (situated on chromosome 14) have been studied extensively in IgA deficient blood donors, but evidence for structural gene deletions could not be found (32). Associations between IgA deficiency and certain HLA antigens (especially B8 and DR3) have been shown (33). Aberrations of chromosome 18 and IgA deficiency were found in several cases (2,16). In families of individuals with hypogammaglobulinemia cases of IgA deficiency have been described, and in relatives of patients with selective IgA deficiency, aberrations in other immunoglobulins are not unusual (29,30,34).

1.3.3 Anti-IgA antibodies

An important feature in patients with selective IgA deficiency is the development of anti-IgA antibodies, reported in frequencies up to 40% (16). The reason why a substantial number of IgA deficient individuals develop anti-IgA antibodies and others do not is not clear.
In individual cases development of anti-IgA after transfusion with IgA containing products (blood or gamma-globulin) is well-known, but such a causal relation could not be recognized in large studies. It has been suggested that development of anti-IgA antibodies may be the result of an increased absorption of antigenic substances that are cross-reactive with IgA, analogously to the development of blood group isoagglutinins.

Data upon the question at what age anti-IgA antibodies become manifest are very scarce in the literature.

Anti-IgA antibodies can be class-specific or of limited specificity, for instance only directed against IgA_1 or IgA_2. Anti-IgA is mostly of the IgG class, but IgE antibodies to IgA have been described (35). The clinical importance of presence of anti-IgA antibodies lies in the fact that they may result in hazardous reactions after transfusion of IgA-containing products (36).

The role of anti-IgA antibodies in the pathogenesis and/or maintenance of IgA deficiency is not known. In vitro studies have revealed that anti-IgA, when added to cell cultures, inhibits in vitro IgA synthesis (37,38). However, as most IgA deficient individuals lack anti-IgA antibodies, this in vitro phenomenon is not likely to play an important role. Interesting observations have been reported in mothers with IgA deficiency and circulating anti-IgA antibodies, suggesting that transplacental passage of anti-IgA antibodies of the IgG class may result in IgA deficiency in the progeny, although the underlying mechanism remained obscure (39,40).

1.3.4 Acquired and transient IgA deficiency

IgA deficiency is not always a primary immunodeficiency, but can also develop during treatment with various drugs. Diminished serum IgA levels have been reported in connection with anticonvulsants (phenytoin), and slow-acting antirheumatic drugs (penicillamin, sulfasalazine and gold salts) (41-47). Most drug-induced IgA deficiencies are temporary, or reversible after withdrawal of the drug, although persistent IgA deficiency has been reported (44-47).

Normalization of serum IgA levels in formerly IgA deficient individuals has been described on several occasions and has been termed transient IgA deficiency (48). Generally this phenomenon is found in young children, and merely reflects delayed maturation of the IgA system. For this reason it seems appropriate to keep some reserve in diagnosing selective IgA deficiency under the age of one year. Especially among cases of "partial" IgA deficiency normalization of IgA levels is common (17).

1.4 CLINICAL MANIFESTATIONS IN SELECTIVE IgA DEFICIENCY

1.4.1 General remarks

The clinical manifestations of IgA deficiency are heterogeneous. Most IgA deficient individuals found in community-based studies appeared to be healthy. On the other hand, associations with a variety of diseases including auto-immune disorders, recurrent infections, gastro-intestinal disease and allergic disorders have been described many times (16,49-52). However, associations between selective IgA deficiency and clinical symptoms as outlined below should be considered with some caution, as in most reports patient groups mainly consisted of selected populations, either biased by positive selection as a (healthy) blood donor or negatively biased as a referred patient in hospital-based series (25). Surveys of somewhat unselected patients are scarce, but usually reveal no striking associations between selective IgA deficiency and disease (25).

Symptomatic patients with selective IgA deficiency have in common that clinical manifestations mostly can be related to the absence of IgA in the secretions. Secretory IgA is the major immunoglobulin in secretions, acting in the protection of mucosal surfaces against invading micro-organisms and preventing penetration of toxins and allergens. So absence of IgA can facilitate infections involving the mucous membranes of the upper and/or lower respiratory tract, or of the gastro-intestinal tract. It is also conceivable that the elevated absorption of foreign antigens may result in an increased incidence of allergic and auto-immune diseases. Since secretory IgA is so clearly thought to be an important part of the local mucosal immune system, it is still unclear why the majority of individuals with selective IgA deficiency are reported to have no complaints whatsoever. Generally this is attributed to compensatory mechanisms in other areas of the immune system, for instance substitution of other immunoglobulin classes for IgA in secretions (16).

1.4.2 Recurrent infections and its association with concomitant IgG subclass deficiencies

The majority of symptomatic IgA deficient patients suffer from frequent infections, mainly of the respiratory tract including middle ear and paranasal sinuses (16,49-52). Infections are mostly mild to moderately severe. The incidence of severe life-threatening infections is not increased in patients with selective IgA deficiency.

Concomitant deficiency of IgG subclasses in individuals with "selective" IgA deficiency was first reported by Oxelius et al. (19), and was found to predispose to more serious and more frequent (respiratory tract) infections. Thereafter this finding has been confirmed in numerous other studies. Björkander et al. reported bronchiectasies and impaired lung function in combined IgG subclass-IgA deficiency (53). IgG subclass deficiency associated with IgA deficiency mainly refers to IgG_2 deficiency and to a lesser extent IgG_4 deficiency. Combined IgA-IgG_2-IgG_4 deficiency is not uncommon. Since antibody activity against polysaccharide antigens resides predominantly within the IgG_2 subclass (54,55), this explains the increased rate of sinopulmonary infections with polysaccharide capsulated bacteria as Haemophilus influenzae and Streptococcus pneumoniae. The clinical relevance of concomitant IgG_4 deficiency remains debatable. Due to the very broad spread in childhood, reference values for IgG_4 are not available, and up to 8% of healthy children have no detectable IgG_4 (56). It is thought that recurrent infections in individuals with IgG_4 deficiency are more likely to be associated with coexisting IgA and/or IgG_2 deficiency (57).

1.4.3 Gastro-intestinal diseases

Chronic infections of the gastro-intestinal tract are rare in selective IgA deficiency (58), suggesting that compensatory mechanisms could be operating in the intestine (59).

Reports of other gastro-intestinal disorders associated with selective IgA deficiency are various. They include pernicious anemia (60), ulcerative colitis (61), Crohn's disease (61), giardiasis (62), and nodular lymphoid hyperplasia (63). Among patients with coeliac disease increased frequencies of IgA deficiency have been reported (58,64). The response to gluten-free diet usually is good, and comparable to that of individuals who are not IgA deficient.

Anti-bovidae antibodies of the IgG class are often found in serum of IgA deficient individuals (64). Cow's milk precipitins have been reported in frequencies up to 75% (65), and can lead to the formation of (possibly harmful) circulating immune complexes (66).

1.4.4 Allergy

Allergic diseases are more often found in IgA deficient individuals (24,65). The most common allergic diseases established include allergic rhinitis, atopic eczema, bronchial asthma and urticaria. Allergic diseases in selective IgA deficiency appear to have a more chronic course and are less associated with seasonal influences (16). Treatment can be more difficult because of a greater incidence of respiratory tract infections.

1.4.5 Auto-Immune disorders

The auto-immune diseases most commonly associated with IgA deficiency are rheumatoid arthritis and systemic lupus erythematosus (16,50,67). Other with auto-immunity related disorders reported in patients with selective IgA deficiency include pernicious anemia (60), ulcerative colitis (61), Crohn's disease (61), dermatomyositis (61), pulmonary haemosiderosis (64), thyreoiditis (68), chronic aggressive hepatitis (69), Addison's disease (70), Sjögren syndrome (71) and idiopathic thrombocytopenic purpura (71). Presence of auto-antibodies (without manifest disease) against parietal cells, basement membrane, mitochondria, DNA, thyroglobulin, collagen and smooth muscle have been reported in IgA deficient individuals (64,72-74).

Buckley found relatively low frequencies of auto-immune disorders in children with selective IgA deficiency (29). However, in adults associations with auto-immune disorders are thought to be sufficiently frequent to warrant periodic investigation for auto-immunity in all IgA deficient patients (16).

1.5 PATHOGENESIS OF IgA DEFICIENCY

1.5.1 Introduction

Most investigators agree upon a developmental defect in B cell maturation towards IgA-producing plasma cells as the major cause of selective IgA deficiency. The cellular and molecular mechanisms involved in the regulation of lymphoid cells to differentiate towards IgA-producing plasma cells and in the control of their characteristic distribution in mucosal tissues are only partially understood (3). IgA plasma cells represent the endpoint of isotype-specific B cell differentiation. This process is influenced at various stages by T cells and/or T cell-derived cytokines (Figure 1.2). IgA production and secretion into serum and secretory fluids are the net result of a complex network of interacting cells. Most investigations on this matter have been done in the murine system, and application of the results to humans has to be done with some caution (3,75). Mature B cells ($sIgD^+$,$sIgM^+$) switch to surface IgA positive ($sIgA^+$) cells either by direct or successive pathways (3). This process is thought to be influenced by T switch cells, environmental antigens and/or interleukin (IL)-4 (3,76). IL-5 induces activated $sIgA^+$ B cells towards proliferation and clonal expansion (3,76,77). Finally, IL-6 (and perhaps IL-5) induces differentiation of $sIgA^+$ B cells into IgA-secreting plasma cells (78).

In several studies however, the number of $sIgA^+$ B cells in peripheral blood in IgA deficient individuals was found to be normal (79-81). In contrast to these findings Conley and Cooper reported decreased numbers of $sIgA^+$ cells (82). The $sIgA^+$ cells in their study were not only decreased in number, but also stained for sIgM and sIgD, indicating an immature state of differentiation (Figure 1.2). Compared to normal individuals the number of these $sIgA^+$-$sIgD^+$-$sIgM^+$ cells was increased. It was suggested that B cell maturation in selective IgA deficiency is arrested in a very early stage of differentiation (82). Decreased numbers of $sIgA^+$ cells have also been reported by others (83-85), but in these studies B cells were not investigated with double-staining techniques.

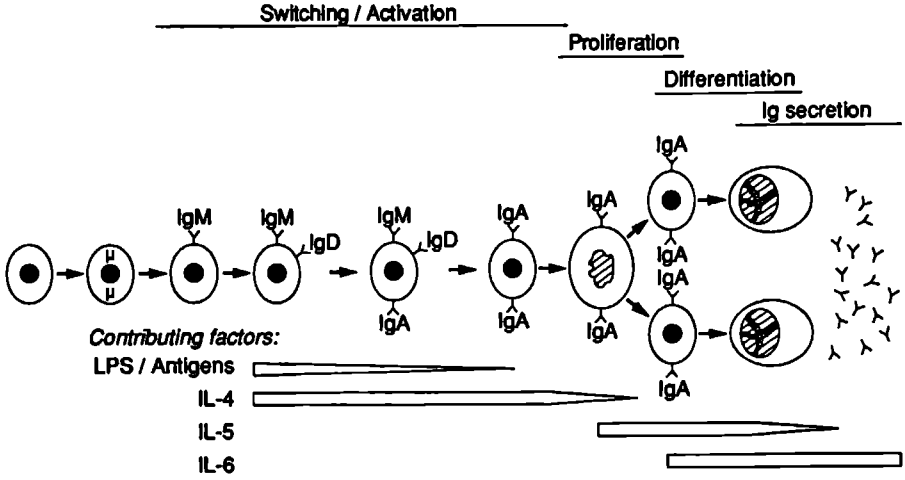

Figure 1.2: *Regulation of B cell differentiation from lymphoid stem cell to IgA-producing plasma cell. The figure is limited to the IgA B cell lineage. The stages at which T cells directly influence the differentiation pathway are not fully known.*

1.5.2 In vitro studies

In many in vitro studies the capacity of peripheral blood mononuclear cells (PBMC) from IgA deficient patients to differentiate into IgA-producing plasma cells has been investigated. In an early report pokeweed mitogen (PWM)-driven cell cultures resulted in normal amounts of IgA-containing plasma cells and normal IgA secretion (86). Waldmann et al. reported a secretory blockade in IgA-producing plasma cells (87). However, in most other in vitro studies PBMC of IgA deficient individuals were unable to produce IgA (81,83-85,88-92).

The cellular basis underlying selective IgA deficiency has been investigated in many studies (80-92). Most studies were limited to small numbers of patients, and populations were not always well-defined with regard to age, mode of inheritance and clinical manifestations.

Interpretation and comparison of the results is hampered by the fact that often different methods were used. In early reports PBMC of IgA deficient individuals and controls were mixed and cultured together in PWM-driven cell cultures. PBMC of some patients were found to depress IgA production by PBMC of controls, but the nature of this suppressive activity became not clear (87-89). In later studies PBMC of IgA deficient individuals and of healthy controls were separated into non-T and T cell fractions (81,83-85,90-92). The non-T and T cell fractions of IgA deficient individuals were cocultured with counterpart cell fractions of controls, thus enabling the separate investigation of B and T cell functions. It was recognized that in some patients with selective IgA deficiency T cell regulatory disturbances existed (90,91). In efforts made to distinguish T helper and suppressor activity different methods have been used: irradiation of the T cell fraction (81,85), depletion of $CD4^+$ or $CD8^+$ cells by complement-mediated lysis (81,84), addition of hydrocortison (84,90,91) or treatment with mitomycin C (92). The coculture studies that enabled appropriate distinction between B and T cell functions and that were methodically comparable showed heterogeneous results (Table 1.1). So, the cellular basis of selective IgA deficiency has often been found to be a pure arrest in B cell maturation, but excessive T suppressor function, diminished T helper function, and combinations of B and T cell abnormalities have also been reported.

Table 1.1: Results of comparable coculture studies investigating the cellular basis of selective IgA deficiency.

		numbers of patients				
Study	(Ref)	B	Ts	Th	B+Ts	B+Th
Oen et al.	(81)	12	4	2		
Cassidy et al.	(83)	8				
Klemola et al.	(84)	19				2
Inoue et al.	(85)	5	5	1	3	
Shinomya et al.	(92)	5	1			

B = defective B cell function
Ts = excessive T suppressor function
Th = diminished T helper function

1.6 AIM OF THE STUDY

In the past decades much has already been written about selective IgA deficiency. There have been numerous reports on frequencies of selective IgA deficiency in all kinds of populations, on modes of inheritance, on clinical manifestations, on pathogenic mechanisms, and on associations with other immune disturbances. Although it is generally considered to be the most frequent primary immunodeficiency, the results of studies on the different aspects of selective IgA deficiency are rather heterogeneous. Up to the present there is no good explanation for this heterogeneity. However, most reports shared in common that the populations or patient groups studied were also heterogeneous or biased. In many studies the analysis of results did not take into account differences in sex, mode of inheritance, or presence or absence of clinical manifestations, and selective IgA deficiency was not always well-defined. Only a few studies have reported longitudinal data (17,29,48,93), whereas the majority represented cross-sectional studies. Investigations on pathogenesis were mostly limited to small numbers of patients and different methods were used. In our opinion these considerations could explain at least some part of the heterogeneity of selective IgA deficiency in the literature.

In this thesis an effort was made to investigate the different aspects of selective IgA deficiency in a well-defined and longitudinally documented group of children and adolescents. In the past fifteen years selective IgA deficiency has been diagnosed in a large group of children known to the Department of Pediatrics of the University Hospital Nijmegen. The majority was diagnosed after referral for complaints as recurrent infections or atopic diseases. Four children were diagnosed in the "Nijmegen Growth Study" (9). Family screening for IgA deficiency revealed additional cases in 8 families, and allowed formation of two groups: "sporadic" and "familial" IgA deficiency. Most patients have been carefully followed during regular outpatient visits. The main goal of our studies was to investigate whether division of the patients into "sporadic" and "familial" cases could lessen the well-known heterogeneity of IgA deficient individuals with regard to clinical manifestations as well as to the underlying pathogenic mechanism responsible for selective IgA deficiency.

1.7 OUTLINE OF THE THESIS

The longitudinal clinical manifestations in 25 cases of "sporadic" and 15 cases of "familial" IgA deficiency are described in **Chapter 2**.

In order to investigate the cellular basis of IgA deficiency a system for PWM-driven in vitro immunoglobulin production was made operational. Using coculture techniques with counterpart non-T and T lymphocytes from healthy controls, and applying adapted statistical comparisons it was possible to distinguish defective B cell function, excessive T suppressor function and diminished T helper function. The methods and statistical calculations of the cell culture system are described in **Chapter 3**.

In vitro immunoglobulin production was studied in 22 patients without anti-IgA antibodies, including both "sporadic" and "familial" cases. The results of the in vitro experiments were compared to the clinical manifestations (**Chapter 4**).

The cell culture system was used to investigate the capacity for in vitro immunoglobulin production in four IgA deficient children of two IgA deficient mothers with class-specific anti-IgA antibodies. Three of these children had anti-IgA antibodies themselves. The results of the experiments and the implications on pathogenesis of IgA deficiency in this apparently distinct group of patients are described in **Chapter 5**.

The cell culture system was also used to investigate in vitro immunoglobulin production in three patients with juvenile chronic arthritis who, while treated with aurothioglucose, developed markedly diminished IgA levels (**Chapter 6**).

During the follow-up period the serum IgA levels in the patients were measured with a sensitive technique. In **Chapter 7** the longitudinal course of serum IgA and other immunoglobulin levels are described.

In **Chapter 8** the results and conclusions of the investigations are discussed.

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Chapter 2

CLINICAL MANIFESTATIONS IN SELECTIVE IgA DEFICIENCY IN CHILDHOOD

A Follow-up Report

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2.1 ABSTRACT

Clinical manifestations in 40 children with selective IgA deficiency were studied during a follow-up period of 2-10 years. The patients were divided into two groups: group I consisted of 25 children with "sporadic" IgA deficiency and group II of 15 children with "familial" IgA deficiency. Respiratory tract infections including otitis media were frequent in both groups. Concomitant IgG_2-IgG_4 deficiency was found in two patients in group I. Longitudinal serum IgG levels were elevated significantly in both groups. Atopic complaints were observed in 10 children of the "sporadic" group, but only in two of the "familial" group. However, elevated serum IgE levels were more often found in group II. Two children of group I were mentally retarded and chromosomal examination showed abnormalities in both. Anti-IgA antibodies were detected in one child in group I and three children in group II. These three patients had an IgA deficient mother with class-specific anti-IgA antibodies. Concomitant IgG_4-IgE deficiency was found in all four.

2.2 INTRODUCTION

Selective IgA deficiency is generally considered to be the most common primary immunodeficiency in man, occurring in frequencies varying from 1:300 to 1:3000, depending on screened population and the applied definition (1,2). The clinical relevance of selective IgA deficiency is controversial. It can be found in healthy individuals (e.g. blood donors), but otherwise there are many reports describing an association between selective IgA deficiency and disease, especially recurrent infections, auto-immune disorders, atopic disease and gastro-intestinal diseases (3-5). Simultaneous deficiency of IgA and one or more IgG subclasses has been described, and appears to predispose to more severe and more frequent infections (6,7).

In most cases selective IgA deficiency occurs sporadically, but familial occurrence with autosomal dominant, autosomal recessive or polygenic patterns of inheritance has been reported (2,3).

The aim of our study was to look for possible differences in clinical manifestations between "sporadic" and "familial" cases of selective IgA deficiency, and in this way to try to find an explanation for the well-known heterogeneity observed in IgA deficient individuals.

2.3 PATIENTS AND METHODS

2.3.1 Patients

During a 10-year period selective IgA deficiency was diagnosed in 40 children. Selective IgA deficiency was defined according to Hong & Ammann (8). They include those patients with markedly diminished serum IgA levels (<0.05 g/l), normal total levels of serum IgG and IgM, and no or at most mild abnormalities of T cell function. Diagnosis was only made after the age of one year. Patients with ataxia telangiectasia were excluded from this study. None of our patients used phenytoin, penicillamin or sulfasalazine, agents with known ability to induce secondary IgA deficiency (9-11).

The patients were divided into two groups. Group I consisted of 25 children who, as family studies revealed no other cases of IgA deficiency, were designated as "sporadic" cases (Table 2.1). Two children were mentally retarded and chromosomal examination showed abnormalities: a boy (P.M.) with ring-chromosome 22 without further

complaints, and a girl (E.vdB.) with ring-chromosome 18 who was suffering from chronic aggressive hepatitis and died at the age of seven. Four children were diagnosed in the "Nijmegen Growth Study" (12). Group II consisted of 15 children with "familial" IgA deficiency who all had a sib with selective IgA deficiency (Table 2.2). The sibling of one of our patients was excluded from this study because of insufficient follow-up. Four children belonging to two families had a mother with IgA deficiency and class-specific anti-IgA antibodies. One of them (Y.T.) developed normal serum IgA levels during his first year of life (13), but at re-examination at the age of five was found to be IgA deficient.

Out-patient follow-up visits took place at least 2-3 times yearly for a period of 2 to 10 years; serum immunoglobulin levels were measured, and clinical information was carefully recorded. Recurrent infection was defined according to Plebani et al. (5) as the occurrence of at least seven episodes of infection in one year of follow-up; or five or more episodes in each of two years of follow-up, or three or more episodes in each of 3 or more years of follow-up. Allergic rhinitis, asthma or atopic eczema were classified as atopic disease.

2.3.2 Serum immunoglobulin measurements

IgG, IgA and IgM levels were determined by single radial immunodiffusion modified by Van Munster et al. (14) till 1979, and later by laser-nephelometry. All values were calibrated against WHO standard serum No. 67/97. Class-specific anti-IgA antibodies were determined by a titration-radioimmunoassay as previously described (15). IgG subclass levels were determined by radial immunodiffusion (14) and calibrated against standard serum containing IgG_1 6.2 g/l; IgG_2 2.4 g/l; IgG_3 0.64 g/l and IgG_4 0.46 g/l (HOO-020, The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). IgG_1 < 3.1 g/l, IgG_2 < 0.48 g/l at the age of 2-5 years and < 0.72 g/l afterwards, and IgG_3 < 0.16 g/l (P_3 values according to a recent study of Plebani et al. (16)) were considered as an indication of deficiency of the respective IgG subclass. IgG_4 levels below 0.046 g/l were considered as an indication of IgG_4 deficiency.

IgE levels were determined by a radioimmunoassay. Lowest limit of detection was 12 ug/l (5 IU/ml). Undetectable values were considered as an indication of IgE deficiency. Serum IgE levels in our patients were compared with those provided by Kjellman (17). Values above +1 SD

were designated "high". In Kjellman's study IgE levels above +1 SD were correlated with an increased incidence of atopic complaints (17).

2.3.3 Statistical analysis

The IgG and IgM levels of the patients were compared with the population growth standards obtained in the "Nijmegen Growth Study" (12). Only children with at least two measurements between 3.5 and 14.5 years of age were admitted. In case of related children only one of them was randomly chosen for analysis.

Assuming log normal distribution for IgG and IgM levels and making use of age and sex adjusted means and standard deviations of the healthy population, for each child the average z of the standardized values of the logarithmically transformed measurements was determined. For testing the null hypothesis that the IgG and IgM levels of IgA deficient children do not deviate from those of normal children, approximate chi square tests were applied; the number of children as the number of degrees of freedom and the sum of the squared values z^2 as the test statistic.

2.4 RESULTS

2.4.1 Clinical findings

Infections: In both groups the main clinical manifestations at diagnosis and during follow-up were recurrent respiratory tract infections (Tables 2.1 and 2.2). Recurrent otitis media was observed in 12 children of group I and in 8 children of group II. Implantation of Fowler drains was performed in half of the cases. Mostly the infections tended to become milder during the follow-up period. Severe infections were rare at diagnosis and during follow-up. The two children with recurrent urinary tract infections (E.D. and Ba.B.) had vesico-ureteric reflux. From the 12 children found to be selectively IgA deficient in family studies or in the "Nijmegen Growth Study" 9 were asymptomatic at diagnosis. Only 4 of them remained asymptomatic during the entire follow-up period. The others all developed complaints during follow-up, mainly consisting of recurrent otitis media.

Atopic diseases: Atopic complaints were also frequently found (Tables 2.1 and 2.2). The incidence was higher in the "sporadic" group: 10 children with atopic manifestations in group I versus 2 in group II. Severe forms of asthma or atopic eczema did not occur.

Gastrointestinal problems: Gastrointestinal complaints were reported in several patients of both groups. Two children of group II had coeliac disease at diagnosis of selective IgA deficiency. Both responded well to a gluten-free diet. In patient M.M. a normal diet was reinstituted after two years without recurrence of coeliac disease. One patient developed Crohn's disease at the age of twenty. Another patient developed bloody stools due to lymphofollicular hyperplasia at the age of seventeen.

2.4.2 Immunological findings

In most patients studied longitudinal serum IgG levels (follow-up period: 2-10 years) were significantly elevated ($p < 0.001$) compared to our age and sex specific normal values (12). Serum IgM levels were within, or slightly above, the normal range ($p = 0.19$). Between the "familial" and "sporadic" group no differences were observed in serum IgG and IgM levels. IgG subclass levels were determined in 36 patients and in some children concomitant IgG subclass deficiencies were found. Combined IgG_2-IgA deficiency was not observed in our patients. Combined IgG_4-IgA deficiency was observed in eight patients; four in each group. Two of them (in group II) suffered from recurrent lower respiratory tract infections, and 5 children (3 in group I; 2 in group II) had recurrent otitis media. The clinical relevance of the IgG_4 deficiency found in 20% of our patients is debatable. Due to the very broad spread in childhood, IgG_4 reference values are not available, and IgG_4 deficiency is difficult to define (16).

IgG_2-IgG_4-IgA deficiency was found in two boys. One boy was mentally retarded and had a ring chromosome 22; he had neither recurrent infections nor atopic disease. The other boy suffered from a Haemophilus influenzae meningitis and severe respiratory tract infections. During follow-up his IgG and IgM levels declined too, and antibody responses were impaired or negative. He must be regarded as a case of late-onset hypogammaglobulinemia.

Table 2.1: Clinical manifestations in group I: 25 children with "sporadic" IgA deficiency.

– = no complaints. UTI = urinary tract infections. RA = rheumatoid arthritis.
Recurrent infections: B = bronchitis, O = otitis, OF = otitis with Fowler drains.
Atopic diseases: A = asthma, E = atopic eczema, R = allergic rhinitis.

			Complaints	During follow-up period		
Patient	Age (yr) at diag.	Sex	At diagnosis	0-2 yr	2-5 yr	5-10 yr
B.S.	1	F	A E	A E	A E	A E
R.vdB.	1	M	B	OF	OF	OF
D.G.	2	F	Lymphadenitis	B OF	OF	–
C.W.	2	F	Diarrhoea, O	OF A	–	O
E.D.	2	M	UTI, OF	OF	OF	–
I.A.	3	F	O	O	O R	R
H.B.	3	M	B A E	A E	–	
E.vdB.	3	F	Retardation, Chronic aggressive hepatitis			
P.dG.	4	M	Fever e.c.i.	O	O	–
T.B.	5	M	OF A E	OF A E	A R	A
D.J.	7	M	B	Osteomyelitis,R	O R	O A R
Tj.J.	8	M	B O	–	–	Bloody stools
J.A.	8	M	A E	O A E R		
M.vdB.	8	M	B	–	–	–
B.K.	9	F	Constipation	OF	–	–
M.dK.	9	M	B	B	B	B
P.M.	9	M	Retardation, Pneumonia	–	–	–
V.P.	10	F	RA?	–	–	
C.E.	11	F	–	–	–	
S.vB.	11	M	A	A	A	A
C.S.	13	F	R	R	R	
W.L.	13	M	–	R	R	M.Crohn, R
M.C.	14	F	Hypomagn.,O	–	–	–
R.vW.	16	F	O	–	–	
P.N.	16	M	Meningitis,B O	B O	B	

Table 2.2: Clinical manifestations in group II: 15 children with "familial" IgA deficiency.

– = no complaints. UTI = urinary tract infections.
Recurrent infections: B = bronchitis, O = otitis, OF = otitis with Fowler drains.
Atopic diseases: A = asthma, E = atopic eczema, R = allergic rhinitis.

Patient	Age (yr) at diag.	Sex	Complaints At diagnosis	During follow-up period 0-2 yr	2-5 yr	5-10 yr
R.F.	3	M	Arthralgia	O	–	–
D.F.	2	M	–	O	O	–
Ba.B.	2	M	UTI	–	–	–
Bu.B.	2	F	–	OF	OF	–
T.M.	1	M	–	O	–	–
M.M.	3	M	Coeliac disease	B O	–	–
B.O.	2	M	B	B O A	B A	
N.T.	1	M	–	OF	OF	OF
Y.T.	5	M	–	–	–	
W.L.	6	F	B	B	B	B
A.L.	4	F	–	–	–	–
P.D.	5	F	Coeliac disease	Persistent coeliac disease		
M.D.	7	F	B E	B OF E	B OF E	B OF E
M.J.	7	M	Bloody stools	Bloody stools	–	–
T.J.	6	M	–	–	–	–

Table 2.3: Numbers of patients with atopic manifestations in relation to the serum IgE levels observed. Serum IgE levels were designated "normal" or "high" according to Kjellman (17). Values below 5 IU/ml were considered as an indication of IgE deficiency.

	Group I ("sporadic")			Group II ("familial")		
Serum IgE levels	Deficient	Normal	High	Deficient	Normal	High
Number of patients						
Total	8	12	5	5	4	6
Atopic complaints	3	3	4	0	0	2

High levels of serum IgE were more often found in group II (Table 2.3). Only two of the six children with "familial" IgA deficiency and high IgE levels suffered from atopic complaints. In the "sporadic" patients high serum IgE levels corresponded better with the occurrence of atopic disease. Surprisingly, 3 children in group I with concomitant IgE deficiency suffered from allergic rhinitis. Their serum IgG_4 levels were normal.

During the follow-up period 4 boys (2 in group I; 2 in group II) developed serum IgA levels above 50 mg/l. Although there was considerable variance during the follow-up period and serum IgA levels are still very low compared to normal values, these patients no longer meet the definition of selective IgA deficiency, and could represent cases of transient IgA deficiency. Noteworthy is the fact that HB has an identical twin brother who is not IgA deficient.

In 4 of the 36 children who remained IgA deficient during the follow-up period, development of class-specific anti-IgA antibodies was detected. None of them had received a transfusion with IgA-containing products. Three of these children had a mother with IgA deficiency and anti-IgA antibodies; they developed anti-IgA antibodies at the ages of three, seven and nine, respectively. The fourth patient was a girl in the "sporadic" group referred to our clinic because of convulsions due to hypomagnesemia caused by an excessive renal loss of magnesium. Anti-IgA antibodies in her case were first detected at the age of eighteen. Besides recurrent otitis media she had no other complaints. In all four patients who developed anti-IgA antibodies a concomitant IgG_4-IgE deficiency was detected.

2.5 DISCUSSION

In both groups of children with selective IgA deficiency clinical manifestations appeared to be similar. They mainly consisted of mild respiratory tract infections including recurrent otitis media. The occurrence of recurrent otitis observed in our study was impressive, and as high as the 44% incidence reported by Buckley (3).

Associations between selective IgA deficiency and clinical symptoms should be considered with some caution, as in most reports and in this study too, patient groups mainly consist of children referred for complaints, and are possibly not representative for the total IgA deficient population (18). However, of the twelve children who were not referred

for complaints, the majority also had respiratory tract infections during the follow-up period. In our opinion this finding is a reliable indication of the predominance of recurrent respiratory tract infections in children with selective IgA deficiency. Although recurrent infections were more frequently found in the younger patients (Tables 2.1 and 2.2), follow-up studies revealed that they also occurred at older ages in the children of both groups.

Analysis of longitudinal serum immunoglobulin levels in our patients revealed a statistically significant elevation of IgG in both groups. We think this elevation is caused by the high incidence of infections, and in this way also supports the predominance of recurrent infections in children with selective IgA deficiency proposed above. Combined deficiencies of IgG subclasses and IgA were not frequent in our patients. In group I two boys with IgG_2-IgG_4-IgA-IgE deficiency were found. One boy surprisingly had no complaints whatsoever. The other boy, suffering from severe recurrent infections, developed late-onset hypogammaglobulinemia, and resembles the patient described by Morell et al. (7). Selective IgA deficiency is thought to be a condition that predisposes to atopic disease (4). We found a high incidence of atopic complaints in group I, but not in Group II. The difference in occurrence of atopic complaints observed between both groups may be due to the fact that most of the patients in group I were referred because of complaints, whereas half of the patients in group II were not referred. However, only five children of group I were referred for atopic complaints. One atopic patient was found in the "Nijmegen Growth Study" (CS), and in four children of group I atopic complaints became manifest during follow-up. Perhaps patients with "sporadic" IgA deficiency are more prone to develop atopic complaints. No association could be established between height of serum IgE levels and the incidence of atopic complaints. In group II the incidence of atopic complaints was low despite high serum IgE levels. In group I three children with normal serum IgE levels had asthma and/or eczema, and three children with IgE deficiency even suffered from allergic rhinitis.

Four boys showed a rise in serum IgA levels above 50 mg/l and we think they represent cases of transient IgA deficiency. Two of them were asthmatic (Table 2.1), and dyspnoeic attacks became less frequent. Patient DF was asymptomatic at diagnosis (Table 2.2), but later developed recurrent otitis despite the rise in serum IgA.

Chromosomal abnormalities in selective IgA deficiency with emphasis on chromosome 18 (deletion, ring formation) have been

reported earlier (4). In our patients chromosomal analysis was performed in sixteen children (19). In two of them chromosomal abnormalities (ring 22 and ring 18) were found, and both children were mentally retarded. In mentally retarded children with selective IgA deficiency chromosomal analysis is strongly recommended.

In four children class-specific anti-IgA antibodies were demonstrated. Three of them had an IgA deficient mother with anti-IgA antibodies. The possibility of induction of selective IgA deficiency by transplacental passage of maternal anti-IgA antibodies has been suggested (13). Our observations indicate that the children also tend to develop anti-IgA antibodies before puberty. The girl with hypomagnesemia developed anti-IgA antibodies only after puberty at the age of eighteen. Concomitant IgG_4-IgE deficiency was found in all four children. This could suggest the possibility of a predominance for development of anti-IgA antibodies in IgG_4-IgE-IgA deficient individuals. A higher frequency of anti-IgA antibodies in IgG_2-IgA deficient patients compared to isolated IgA deficient patients has been reported (20), but no information was given about the time of first occurrence of anti-IgA antibodies. Perhaps periodic screening for anti-IgA antibodies before puberty can be reserved for IgA deficient children of IgA deficient mothers with detectable anti-IgA antibodies and for IgA deficient children with concomitant IgG subclass and/or IgE deficiency.

2.6 REFERENCES

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Chapter 3

IN VITRO IMMUNOGLOBULIN PRODUCTION BY LYMPHOCYTES IN A POKEWEED MITOGEN DRIVEN CULTURE SYSTEM:

Methods and Calculations

3.1 INTRODUCTION

As outlined in Chapter 1, investigations on the cellular basis of selective IgA deficiency have been described in numerous reports over the past fifteen years (1-12). In most reports the capacity of peripheral blood mononuclear cells (PBMC) of IgA deficient individuals for pokeweed mitogen (PWM)-driven in vitro IgA production was studied. In some studies PBMC have been divided into non-T and T cells, usually by rosetting techniques (5,6,8-12). Cocultures of patient non-T or T cell fractions with counterpart cell fractions of healthy controls enabled the discrimination of the role of B and/or T cell abnormalities in the pathogenesis of selective IgA deficiency. Low-dose irradiation of T cells has been demonstrated to abolish T suppressor function without affecting T helper activity (13). Another method for reliable distinction of T suppressor and T helper function in the T cell fraction is the use of cell fractions depleted in $CD4^+$ or $CD8^+$ cells by complement-mediated lysis using anti-T cell antibodies (11,12).

In order to investigate the cellular basis of selective IgA deficiency in our patients, an in vitro cell culture system comparable to those used in earlier reports (9,11) was made operational in our laboratory. Patient B and T cell functions were studied by separating PBMC in non-T and T cell fractions, and culturing them with counterpart fractions of healthy controls. T suppressor and T helper activity were functionally distinguished by evaluating the effects of irradiation of the T cell fractions. The amounts of IgA and IgM secreted into the culture supernatants were measured. The cultured cells were examined for cytoplasmic IgA content in order to investigate the possibility of a secretory blockade for IgA as described by Waldmann et al. (3).

3.2 THE CELL CULTURE SYSTEM

3.2.1 Cell preparation

PBMC were isolated from defibrinated venous blood of IgA deficient children (40-60 ml) and healthy controls (100-150 ml) by density gradient centrifugation on a layer of Ficoll-Isopaque (density 1.077). The defibrinated blood sample was diluted 1:1 with TRIS-buffered Eagle's Minimal Essential Medium (MEM, GIBCO Europe,Paisley,Scotland) containing 100,000 U Penicillin and 100 mg Streptomycin per liter (MEM-

TRIS). Two volumes of diluted blood were pipetted on one volume of Ficoll-Isopaque. Centrifugation was carried out at 1000xg for 20 minutes at 20°C. The obtained PBMC from the interphase were washed two times with 5% fetal calf serum (FCS, Seralab,Crawley Down,England) in MEM-TRIS. Erythrocytes were lysed for 10 minutes at 37°C with TRIS-buffered ammoniumchloride solution (0.14 mol/l, pH 7.2).

Separation into T and non-T cell fractions was performed by a rosetting technique with 2-aminoethyl isothiouroniumbromide hydrobromide-treated fresh sheep red blood cells (AET-SRBC). The AET-SRBC suspension was prepared by first washing the sheep erythrocytes 5 times with sterile saline, then mixing 1 volume of SRBC suspension with 4 volumes of AET solution (4 grams of AET per 100 ml, pH 9.0) and then incubating the mixture for 15 minutes at 37°C. After four more washings with sterile saline a 1% AET-SRBC suspension in 20% FCS/MEM-TRIS was prepared. Equal volumes of a cell suspension containing $4x10^6$ PBMC per ml 5% FCS/MEM-TRIS and the cell suspension of 1% AET-SRBC in 20% FCS/MEM-TRIS were mixed and incubated for 15 minutes at 37°C, centrifuged and left overnight at 4°C. The next day the cell pellet was gently resuspended and centrifuged during 20 minutes at 1000xg on a layer of Ficoll-Isopaque (density 1.085). Interphase cells (non-T cells) and cell pellet (T cells) were both washed two times with 5% FCS/MEM-TRIS, and SRBC were lysed with ammoniumchloride solution.

The T and non-T cell fractions thus obtained were resuspended in a cell culture medium containing 25% FCS in RPMI 1640 (GIBCO) with addition of 2.0 mmol/l glutamine, and penicillin-streptomycin as described above.

3.2.2 Cell cultures

The cultures always consisted of $3x10^5$ cells in 1 ml of culture medium, and were incubated in round-bottomed tubes in triplicate for 9 days at 37°C in a 5% CO_2, high-humidity incubator.

Unseparated PBMC of IgA deficient children and of controls were cultured in order to relate the number of cytoplasmic IgA-containing cells to the amount of IgA secreted in the culture supernatant.

Non-T and T cell fractions of IgA deficient children and of controls were either autologously recombined, or cocultured with their respective counterpart cell fractions (Table 3.1). Cocultures consisted of $1.5x10^5$

non-T cells and $1.5x10^6$ T cells, without correction for the number of monocytes. Each coculture, except in most experiments the recombination of patient non-T cells and control T cells, was also performed with irradiated T cells (3000 rads from a Cesium-sourced Gammacell 1000B blood cell irradiator; Isomedix Inc, Parsippany,NJ) to eliminate T suppressor function. Cells were cultured with and without stimulation by pokeweed mitogen (PWM, GIBCO Laboratories, Grand Island,NY) in a final dilution of 12.5 micrograms/ml medium. Supernatants were collected and stored at -20°C until determination of immunoglobulin (Ig) content. In most in vitro Ig-production experiments two children with selective IgA deficiency were examined at the same time, employing the same healthy control for comparison and coculture techniques.

Table 3.1: Cocultures performed within one patient-control combination.

Control non-T cells	+	Control	T	cells
	+	Control	T*	cells
	+	Patient	T	cells
	+	Patient	T*	cells
Patient non-T cells	+	Patient	T	cells
	+	Patient	T*	cells
	+	Control	T	cells
	+	Control	T*	cells

T* = irradiated T cells

3.2.3 Immunoglobulin assays in the culture supernatant

Supernatant IgA and IgM contents were measured in duplicate after nine days of culture using a class-specific enzyme-linked double-antibody immunosorbent assay (ELISA). Briefly, polystyrene 96-well, flat-bottomed microtiter plates (NUNC,Roskilde,Denmark) were coated overnight at 4°C with rabbit anti-human IgA or IgM gammaglobulin fraction (DAKO,Glostrup,Denmark) in 0.05 M sodiumbicarbonate (pH 9.6). After washing with phosphate-buffered saline (PBS), containing 0.05% Tween 20, 100 microliter aliquots of tissue culture supernatant samples or reference serum diluted in tissue culture medium were added to the wells, and the plates were incubated for 90 minutes at 37°C. Next, the plates were washed again with PBS-Tween 20 and incubated for 90 minutes at 37°C with horse-radish peroxidase (HRP)-labeled rabbit anti-human IgA or IgM (DAKO). The plates were then washed again, and H_2O_2 with o-phenylenediamine-di-HCl as chromogene was added in citrate-phosphate buffer (pH 5.0). Color development was monitored spectrophotometrically at 492 nm by using an automated reader (Titertek Multiskan, Flow Labs, Maclean,VA). Values were expressed in terms of nanograms of Ig per milliliter of culture supernatant. Lowest limit of detection was 10 ng/ml for IgA and 20 ng/ml for IgM. The variation of the duplicate ELISA Ig measurements ranged 3-6% for both IgA and IgM. The variation in Ig production within the triplicate set of cultures ranged 15-30% for both IgA and IgM.

3.2.4 Detection of cytoplasmic IgA

After removal of the supernatant, the cells of the triplicate set of culture tubes containing unseparated PBMC were combined. They were washed three times with a solution containing 60 ml PBS, 20 ml 20% bovine serum albumin (BSA, Organon Teknika,Oss,The Netherlands) and 2 ml 5% EDTA-PBS (pH 7.8). The cells were fixed with 0.5% formaldehyde after the first washing procedure. Cytospin slides were made in the usual way, and cytoplasmic IgA was stained with fluorescein-labeled goat $F(ab')_2$ antihuman IgA (Kallestad,Austin,TX). IgA-containing cells were visualized using a Zeiss fluorescense microscope and expressed as a percentage of the total cell amount (at least 1000 cells were counted).

3.2.5 Determination of cell surface markers

Lymphocytes isolated from defibrinated peripheral blood by the Ficoll-Isopaque density gradient method were suspended in PBS with 1% BSA (PBS-BSA).

Determination of surface Ig^+ cells:

After washing in PBS-BSA at 4°C the cells were pre-incubated for 30 minutes at 37°C to remove adsorbed serum immunoglobulins. After two more washings in PBS-BSA at 4°C, 50 microliter cell suspension (or $2x10^6$ cells) were mixed with 100 microliter diluted tetramethyl-rhodamine isothiocyanate (TRITC)-labeled polyclonal goat $F(ab')_2$ antihuman total immunoglobulins antiserum (Kallestad). Incubation was performed at 4°C during 20 minutes. After incubation the cells were washed 3 times with PBS-BSA + 0.02% sodium-azide at 4°C for 30 minutes and placed on a slide with one drop of buffered glycerol. The slides were examined under a Zeiss Standard microscope equipped with a Ploem-type vertical illuminator, a high performance mercury lamp HBO 50 (Osram), and a suitable filter combination.

Determination of $CD4^+$, $CD5^+$ and $CD8^+$ T cells:

After three washings in PBS-BSA at 4°C, 50 microliter cell suspension (or $2x10^6$ cells) were mixed with 100 microliter diluted monoclonal antiserum (Leu-1 (Becton Dickinson,Sunnyvale,CA) for $CD5^+$-cell determination, OKT4 and OKT8 (Coulter Immunology,Hialeah,FL) for $CD4^+$ and $CD8^+$ cells, respectively). After three washings with PBS-BSA at 4°C a second incubation was performed at 4°C for 30 minutes with 100 microliter diluted fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin antiserum. The cells were then washed three times with PBS-BSA + 0.02% sodium-azide at 4°C for 30 minutes and thereafter placed on a slide with one drop of buffered glycerol and examined by immunofluorescence microscopy as described above.

3.3 CALCULATIONS

After averaging the duplicate Ig measurements in the culture supernatant the results of certain cocultures within one patient-control combination were compared. Undetectable values were replaced by 5 ng/ml. The comparisons of cocultures used within one patient-control combination and the conditions set to define the pathogenic mechanisms of selective IgA deficiency are shown in Table 3.2.

Table 3.2: Comparisons of certain cocultures within one patient-control combination used to distinguish different pathogenic mechanisms of selective IgA deficiency.

COMPARISONS				CONDITIONS SET FOR PATHOGENIC MECHANISMS				
				B	Ts	Th	B+Ts	B+Th
1	= PnonT + CT	≤	CnonT + CT	+	−	−	+	+
2	= CnonT + PT	≤	CnonT + CT	−	+	+	+	+
3	= CnonT + PT*	≤	CnonT + CT	−	−	+	−	+
4	= CnonT + PT*	≥	CnonT + PT	−	+	−	+	−
5	= PnonT + PT*	≥	PnonT + PT	−	+	−	+	− (only for IgM)

B = defective B cell function
Ts = excessive T suppressor function
Th = diminished T helper function
C = control
P = patient
nonT = non-T cells
T = T cells
T* = 3000 rad irradiated T cells
\+ = p-value ≤ 0.0125 for IgA (total p ≤ 0.05)
p-value ≤ 0.0100 for IgM (total p ≤ 0.05)
− = not significant

Because of considerable differences in the variation of the values one-sided t-tests modified according to Satterthwaite were used for the comparisons of cocultures within one patient-control combination (TTEST-SAS procedure). As shown in Table 3.2, full distinction of the possible pathogenic mechanisms necessitated multiple comparisons: four in case of in vitro IgA production and five in case of in vitro IgM production. The Bonferroni adjustment was applied to determine significance levels for analysis involving multiple comparisons. This implied a significance level of $p = 0.0125$ for in vitro IgA production (four comparisons) and $p = 0.0100$ for IgM (five comparisons). According to the Bonferroni adjustment (the sum of multiple tests), this results in a total significance level of $p = 0.05$ for the reliability of a pathogenic mechanism concluded from the results of the cocultures within one patient-control combination.

3.4 INTERPRETATION OF THE CELL CULTURE RESULTS

In previous reports defective IgA B cell function, excessive T suppressor function, diminished T helper function, and combinations of B and T cell abnormalities have been described as pathogenic mechanisms underlying selective IgA deficiency (3-12).

Using the cell culture system described above it was possible to evaluate B and T cell functions for in vitro IgA production in our patients. The results of the coculture experiments within one patient-control combination (Table 3.1) can be compared. The expected amounts of in vitro Ig production in the cocultures in case of defective B cell function, excessive T suppressor function, diminished T helper function and combinations of B and T cell abnormalities are shown in Figures 3.1-3.5.

In vitro IgM production was measured simultaneously with in vitro IgA production in all cell cultures. Defective in vitro Ig production can be found in individuals who are so-called non-responders to stimulation with PWM. Another possibility can be the generation of T suppression in cocultures of allogeneic cells, which has been reported to occur in 1% of randomly chosen combinations (13). By measuring simultaneous IgM production in the cell culture supernatants these two possible causes of defective in vitro IgA production can be excluded, as they are not isotype-specific.

3.4.1 Diminished B cell function (Figure 3.1, Table 3.2)

Diminished B cell function (B) was concluded if Ig production by cocultures of patient non-T cells + control T cells was significantly lower than Ig production by recombined control non-T + T cells (comparison 1: PnonT+CT ≤ CnonT+CT: +).

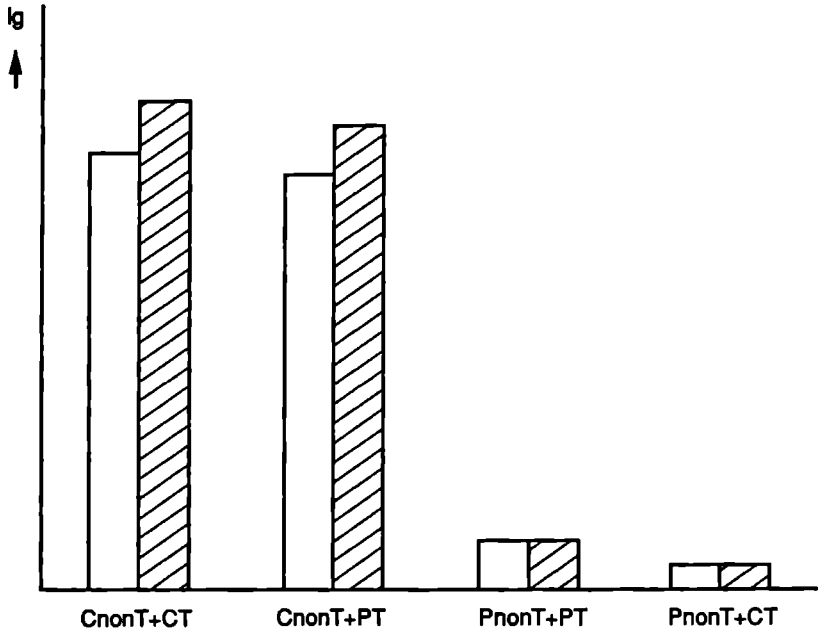

Figure 3.1: *Diminished B cell function. In vitro Ig production by lymphocytes from a healthy control person and a patient with selective IgA deficiency. Cocultures were performed after recombination of equal numbers of non-T and T cells ($1.5x10^5$). C = control; P = patient; open bars represent cocultures with nonirradiated T cells; striped bars cocultures with irradiated T cells.*

3.4.2 Excessive T suppressor function (Figure 3.2, Table 3.2)

Excessive T suppressor function (Ts) was only concluded if the following conditions were all three fulfilled:

1) Ig production by cocultures of control non-T + patient T cells must be significantly lower than Ig production by recombined control non-T + T cells (comparison 2: CnonT+PT $\leq$ CnonT+CT: +);
2) Ig production by cocultures of control non-T + irradiated patient T cells must not be significantly different from Ig production by control non-T + T cells (comparison 3: CnonT+PT* $\leq$ CnonT+CT:-);
3) Ig production by cell cultures containing irradiated patient T cells must be significantly higher than Ig production by cell cultures containing non-irradiated patient T cells (comparison 4: CnonT+PT* $\geq$ CnonT+PT: +; and for IgM production also comparison 5: PnonT+PT* $\geq$ PnonT+PT: +).

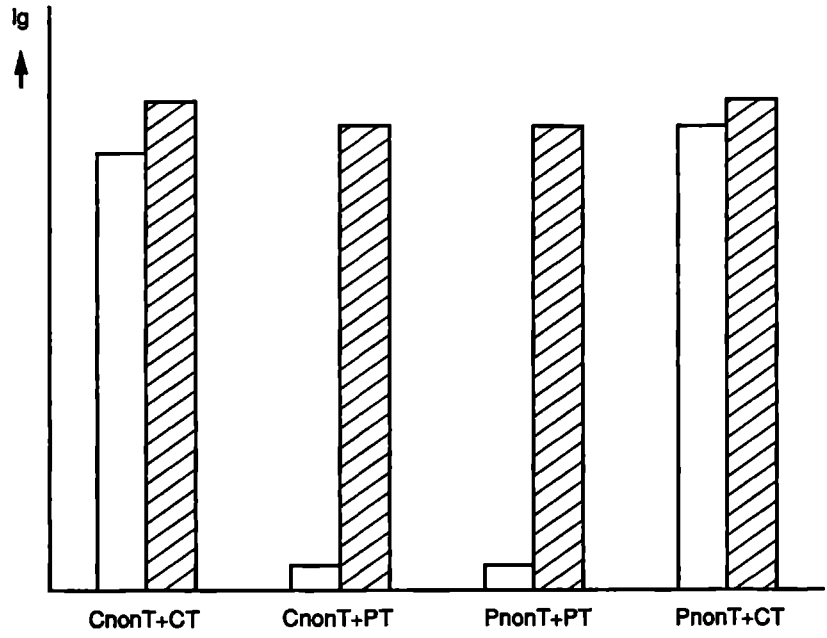

Figure 3.2: Excessive T suppressor function (legend as in Figure 3.1).

3.4.3 Diminished T helper function (Figure 3.3, Table 3.2)

Diminished T helper function was only concluded if the following conditions were all three fulfilled:

1) Ig production by cocultures of control non-T + patient T cells must be significantly lower than Ig production by recombined control non-T + T cells (comparison 2: CnonT+PT $\leq$ CnonT+CT: +);
2) Ig production by cocultures of control non-T + irradiated patient T cells must be significantly lower than Ig production by control non-T + T cells (comparison 3: CnonT+PT* $\leq$ CnonT+CT: +);
3) Ig production by cell cultures containing irradiated patient T cells must not be significantly higher than Ig production by cell cultures containing non-irradiated patient T cells (comparisons 4: CnonT+PT* $\geq$ CnonT+PT: –, and for IgM production also comparison 5: PnonT+PT* $\geq$ PnonT+PT: –).

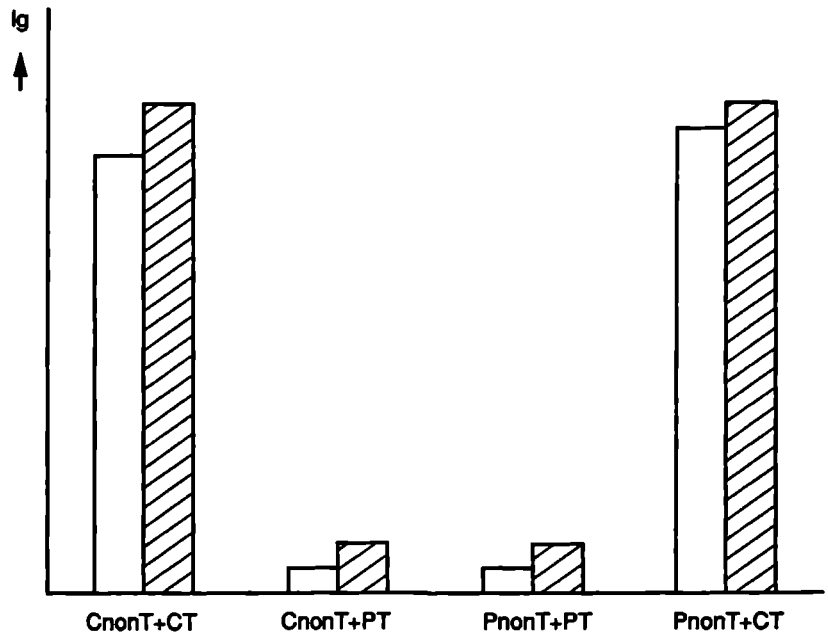

Figure 3.3: Diminished T helper function (legend as in Figure 3.1).

3.4.4 Diminished B cell function combined with excessive T suppressor function (Figure 3.4, Table 3.2)

All conditions set above for both diminished B cell function and excessive T suppressor function must be fulfilled before this could be concluded (comparisons 1: +, 2: +, 3: –, 4: +, and for IgM also 5: +).

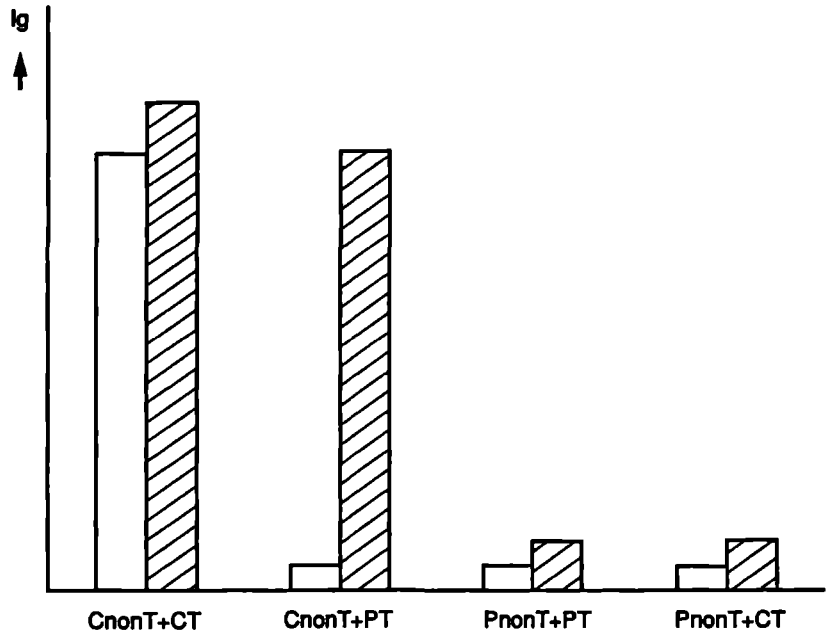

Figure 3.4: *Diminished B cell function combined with excessive T suppressor function (legend as in Figure 3.1).*

3.4.5 Diminished B cell function combined with diminished T helper function (Figure 3.5, Table 3.2)

All conditions set above for both diminished B cell function and diminished T helper function must be fulfilled before this could be concluded (comparisons 1: +, 2: +, 3: +, 4: –, and for IgM also 5: –).

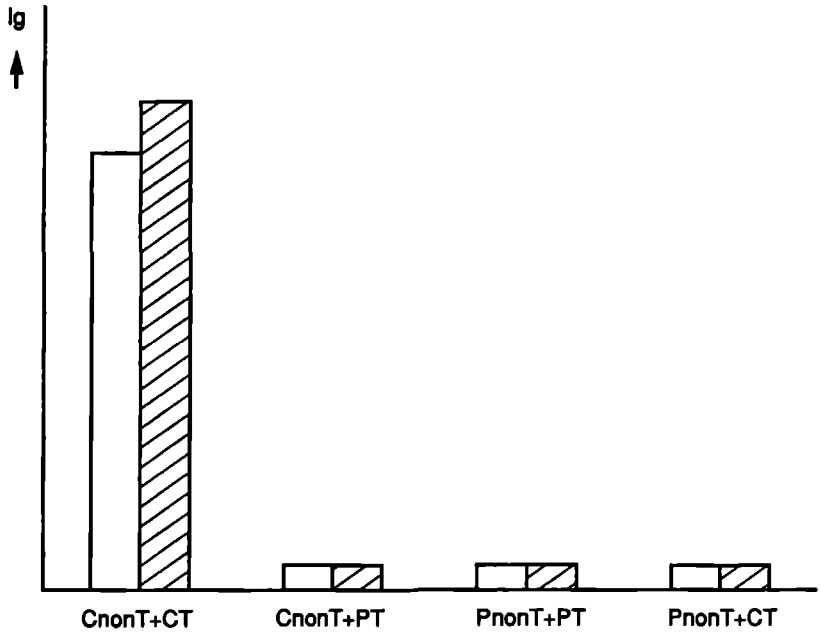

Figure 3.5: *Diminished B cell function combined with diminished T helper function (legend as in Figure 3.1).*

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Chapter 4

IN VITRO IMMUNOGLOBULIN PRODUCTION IN CHILDREN WITH SELECTIVE IgA DEFICIENCY IN RELATION TO CLINICAL MANIFESTATIONS

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4.1 ABSTRACT

Twentytwo children with selective IgA deficiency (14 "sporadic" and 8 "familial") were investigated for pokeweed mitogen-driven in vitro immunoglobulin production. A defect in IgA production by peripheral blood mononuclear cells was established in all patients but one. In four children also in vitro IgM production was below the range of healthy controls. Cocultures performed with patient and/or control non-T and T cells revealed a B cell defect for in vitro IgA production in all but one of the patients. Nine patients had a pure B cell defect, but in 12 patients additional T cell regulatory disturbances could be established. IgA-specific excessive T suppressor function was found in 3 patients. IgA-specific diminished T helper function was found in 4 patients, and diminished T helper function for both in vitro IgA and IgM production could be demonstrated in 5 patients. Clinical manifestations in children with pure B cell defects or with B cell defects and additional IgA-specific T cell abnormalities were comparable: mainly mild respiratory tract infections and atopic complaints. It was noted that most of the patients with more severe infections or with complaints other than recurrent infections or atopy also had disturbances in in vitro IgM production.

4.2 INTRODUCTION

Selective IgA deficiency is generally considered to be the most common primary immunodeficiency. Clinical manifestations of selective IgA deficiency vary from none at all to associations with the occurrence of various diseases as auto-immune disorders, recurrent infections, gastro-intestinal complaints and atopic disease (1,2). Simultaneous occurrence of IgG subclass deficiencies (especially IgG_2 and/or IgG_4) in patients with selective IgA deficiency have been reported, and were related to a high rate of infections (3,4). Investigations on the primary immune response of patients with selective IgA deficiency revealed general disturbances in humoral and cellular immunity to newly encountered antigens (5). Most cases of selective IgA deficiency are "sporadic", but familial occurrence with various patterns of inheritance is well-known (1,2,6).

The results of studies on the cellular basis of selective IgA deficiency are diverse. Waldmann et al. reported a secretory blockade of IgA producing cells (7). Others attributed selective IgA deficiency to an inability of B cells to differentiate into IgA producing cells (8). In later studies a developmental defect in B cell differentiation was suggested as the major cause, although combinations of B and T cell functional abnormalities have been described in several patients (9-14).

In the present study we investigated the cellular basis of selective IgA deficiency in 22 children. Our study included both "sporadic" and "familial" cases of selective IgA deficiency. Special regard was paid to the clinical manifestations and the possibility of differences in pathogenesis between both groups.

4.3 METHODS

4.3.1 Patients and controls

Twentytwo children and adolescents with selective IgA deficiency were investigated (16 boys and 6 girls). The clinical history has been reported previously (15). Selective IgA deficiency was defined according to Hong and Ammann (16): serum IgA level: < 50 mg/liter, normal serum IgG and IgM levels, and no or at most mild abnormalities of T cell function. Two of the children formerly were IgA deficient, but had developed serum IgA levels just above 50 mg/l shortly before the in vitro experiments were performed. None of our patients had circulating anti-IgA antibodies.

Serum immunoglobulins were measured in all first degree relatives of our patients. Eight children had a sibling with IgA deficiency and were considered as "familial" cases. The 14 children without affected sibs were considered as "sporadic" cases. Age at the time of the cell culture experiments ranged from 3 to 21 years (median 11 years).

The control group of eleven persons mainly consisted of healthy laboratory workers with normal serum immunoglobulin levels and intact in vitro immunoglobulin production in previous pilot experiments.

4.3.2 Serum Immunoglobulin measurements

Serum immunoglobulin levels were determined by laser-nephelometry using a DISC 120 Nephelometer (Hyland,Nivelles,Belgium), and all values were calibrated against WHO standard serum No.67/97. Serum IgA levels below the detection limit of the applied nephelometric technique (50 mg/l), and, if present, the amount of class-specific anti-IgA antibodies were further quantitated with a radioimmunoassay (17).

4.3.3 In vitro experiments

Cell preparation and cell cultures were performed as described in Chapter 3. Briefly, peripheral blood mononuclear cells (PBMC) from patients and controls were isolated by density-gradient centrifugation and separated into T and non-T cell fractions. T and non-T cells from controls and patients were either autologously recombined or cocultured with respective counterpart cells in triplicate PWM-driven cell cultures. Each cell culture except PnonT+CT was also performed with irradiated T cells (3000 rads) to eliminate T suppressor function (18). IgA and IgM concentrations in the culture supernatants were measured by ELISA and expressed as nanograms of immunoglobulin per milliliter supernatant. After nine days of culture the number of cytoplasmic IgA^+-cells was examined in cultures of unseparated PBMC from children with selective IgA deficiency or from healthy controls.

4.3.4 Calculations

For comparison of in vitro IgA production by unseparated PBMC a Wilcoxon-test modified according to Gehan was used. Correlation between in vitro IgA production and the number of cytoplasmic IgA^+-cells was calculated according to Spearman.
A one-sided t-test modified according to Satterthwaite was used for comparison of certain cocultures within one patient (TTEST-SAS procedure). The Bonferroni adjustment was applied to determine significance levels for analysis involving multiple comparisons. This implied a significance level of $p = 0.0125$ for IgA (four comparisons) and $p = 0.01$ for IgM (five comparisons). The conditions set to distinguish defective B cell function, excessive T suppressor function and diminished T helper function are shown in Table 4.1.

Table 4.1: Comparisons of certain cocultures within one patient-control combination used to distinguish different pathogenic mechanisms of selective IgA deficiency (for explanation see also Chapter 3).

COMPARISONS				CONDITIONS SET FOR PATHOGENIC MECHANISMS				
				B	Ts	Th	B+Ts	B+Th
1	= PnonT + CT	≤	CnonT + CT	+	−	−	+	+
2	= CnonT + PT	≤	CnonT + CT	−	+	+	+	+
3	= CnonT + PT*	≤	CnonT + CT	−	−	+	−	+
4	= CnonT + PT*	≥	CnonT + PT	−	+	−	+	−
5	= PnonT + PT*	≥	PnonT + PT	−	+	−	+	− (only for IgM)

B	=	defective B cell function	C	=	control
Ts	=	excessive T suppressor function	P	=	patient
Th	=	diminished T helper function	nonT	=	non-T cells
			T	=	T cells
			T*	=	irradiated T cells
+	=	p-value ≤ 0.0125 for IgA (total $p \leq 0.05$)			
		p-value ≤ 0.0100 for IgM (total $p \leq 0.05$)			
−	=	not significant			

4.4 RESULTS

4.4.1 In vitro IgA production

Unseparated PBMC or autologous recombinations of non-T and T cells

Unseparated PBMC of healthy controls were able to synthesize and secrete IgA in vitro when stimulated by PWM (Table 4.2). After recombination of non-T and T cells of healthy controls, comparable amounts of IgA could be detected in the culture supernatant.

Unseparated PBMC or recombined non-T and T cells of most children with selective IgA deficiency were unable to produce significant amounts of IgA (Table 4.2). At the time the cultures were performed the children MJ and DF turned out to have serum IgA levels around, or just above, the limit of 50 mg/l used to define selective IgA deficiency (Table 4.2). Afterwards, serum IgA levels in both patients remained above 50 mg/l, although far below the lower range of age specific reference values. IgA production by their unseparated PBMC reached higher levels, and in one case (DF) was almost in the range shown by healthy controls. There was a significantly ($p=0.003$) lower IgA production by unseparated PBMC in "sporadic" patients with selective IgA deficiency, compared to "familial" patients. If children with serum IgA levels above 10 mg/l were left out of consideration, the difference still remained significant ($p=0.03$).

Cytoplasmic IgA

The number of cytoplasmic IgA^+-cells correlated well ($r=0.93$; $p<0.001$) with the amount of IgA secreted into the culture supernatant. Compared to healthy controls significantly ($p<0.001$) lower numbers of cytoplasmic IgA^+-cells were found after PWM-stimulated cell culture in children with selective IgA deficiency, leaving out DF (Table 4.2). Comparison of "sporadic" and "familial" cases of selective IgA deficiency revealed significantly ($p=0.01$) lower numbers of cytoplasmic IgA containing cells in the "sporadic" cases. The difference remained significant ($p=0.02$) if children with serum IgA levels above 10 mg/l were left out of consideration.

Table 4.2: Serum IgA levels and in vitro IgA production in selective IgA deficient children and healthy controls.

Patients	serum IgA (mg/l)	cytoplasmic IgA[a] (%)	In vitro IgA production[b] PBMC (ng/ml)	recombined (ng/ml)
Sporadic				
SvB	0.59	0.1	14	16
BK	0.35	0.2	13	12
BS	0.43	0.2	34	41
TjJ	0.65	<0.1	<10	<10
MB	0.80	<0.1	<10	<10
PM	0.23	<0.1	28	31
MvdB	19.41	0.2	90	89
RvdB	2.37	0.1	<10	<10
IA	0.23	<0.1	16	15
PdG	0.53	<0.1	<10	10
ED	11.0	0.6	35	35
WL	0.22	<0.1	10	11
DG	0.28	<0.1	<10	<10
DJ	0.48	0.1	16	<10
Familial				
RF	0.28	0.5	25	27
DF	45.1	2.4	645	478
MM	0.58	0.4	40	27
TM	0.28	0.1	59	21
MJ	75.0	0.7	102	74
TJ	0.48	0.4	95	37
MD	1.50	<0.1	<10	<10
PD	21.2	0.8	248	51
Controls (n=11)				
mean	2900	4.1	1737	1314
range	(1000-5460)	(2-10)	(698-4884)	(502-4403)

[a] The number of cytoplasmic IgA-containing cells after nine days culturing of unseparated PBMC is presented as a percentage of the total cell amount on the cytospin slide.

[b] In vitro IgA production by PBMC or recombined non-T and T cells is shown as calculated mean of each triplicate set of cultures after averaging the duplicate measurements.

Cocultures of non-T and T cells

A pure B cell defect was found in 6 patients (Table 4.3). In 4 of our patients (SvB, BK, RF and DF) no defect for in vitro IgA production could be concluded from the statistical comparisons of certain cocultures (Table 4.3). However, in patients SvB, BK and RF, comparison 1 (PnonT+CT $\leq$ CnonT+CT) was almost significant ($0.0125 < p \leq 0.025$, Bonferroni adjustment for four comparisons: total $p \leq 0.10$). It has to be noted that the variation in the triplicate IgA measurements of the control used in these patients was rather large (Table 4.3). The numbers of cytoplasmic IgA-positive cells after PWM-driven culture (Table 4.2) and also in vitro IgA production in cultures containing patient non-T cells were very low in these three patients (Table 4.3). In our opinion these 3 patients also had an IgA B cell defect. Up to the present their serum IgA levels remained below 50 mg/l. Only patient DF revealed a low-normal number of cytoplasmic IgA-positive cells after PWM-driven culture and demonstrated almost normal in vitro IgA production, in spite of his up till now markedly depressed serum IgA levels.

Table 4.3: Patients without T cell defects for in vitro IgA production.

PATIENTS	IN VITRO IgA PRODUCTION (ng/ml)						
	CnonT+CT	CnonT+CT*	CnonT+PT	CnonT+PT*	PnonT+PT	PnonT+PT*	PnonT+CT
Sporadic							
BS	548 ± 33	121 ± 60	439 ± 70	194 ± 44	41 ± 3	43 ± 8	45 ± 12
TjJ	502 ± 108	2183 ± 437	298 ± 39	1164 ± 106	<10	25 ± 12	<10
MB	995 ± 165	1535 ± 161	585 ± 171	832 ± 145	<10	<10	61 ± 19
PM	1270 ± 96	1718 ± 63	953 ± 98	1274 ± 292	31 ± 8	29 ± 12	159 ± 50
MvdB	4403 ± 537	4703 ± 475	965 ± 360	2586 ± 140	89 ± 18	98 ± 27	260 ± 28
SvB	821 ± 218	380 ± 148	528 ± 110	716 ± 99	16 ± 10	77 ± 25	58 ± 12
BK	821 ± 218	380 ± 148	317 ± 28	112 ± 31	12 ± 4	11 ± 4	141 ± 22
Familial							
MM	788 ± 34	1040 ± 41	655 ± 160	620 ± 24	27 ± 6	<10	106 ± 31
RF	866 ± 257	2031 ± 137	430 ± 39	319 ± 195	27 ± 12	<10	41 ± 27
DF	866 ± 257	2031 ± 137	625 ± 179	378 ± 39	476 ± 64	206 ± 27	508 ± 106

C = control. P = patient. nonT = non-T cells.
T = T cells. T* = 3000 rad irradiated T cells.

In the remaining 12 patients also a B cell defect for PWM-driven in vitro IgA production could be established, but they had disturbances in T cell function as well (Tables 4.4 and 4.5). A B cell defect with co-existence of IgA-specific excessive T suppressor function was concluded in two of the "sporadic" and in one of the "familial" patients (Table 4.4).

Table 4.4: Patients with an intrinsic B cell defect for in vitro IgA production and additional IgA-specific excessive T suppressor function (B+Ts).

PATIENTS	IN VITRO IgA PRODUCTION (ng/ml)						
	CnonT+CT	CnonT+CT*	CnonT+PT	CnonT+PT*	PnonT+PT	PnonT+PT*	PnonT+CT
Sporadic							
RvdB	995 ± 165	1535 ± 181	475 ± 67	783 ± 88	<10	29 ± 4	60 ± 68
IA	4403 ± 537	4703 ± 475	1866 ± 348	3774 ± 96	15 ± 11	24 ± 15	102 ± 37
Familial							
PD	1627 ± 184	1692 ± 296	405 ± 61	1133 ± 181	51 ± 29	111 ± 24	227 ± 40

C = control. P = patient. nonT = non-T cells.
T = T cells. T* = 3000 rad irradiated T cells.

A B cell defect with co-existence of diminished T helper function was concluded in five of the "sporadic" patients and in four of the "familial" patients (Table 4.5). The diminished T helper function was found to be IgA-specific in the two brothers MJ and TJ, and in one patient of family D. In the "sporadic" patient PdG comparison 2 was statistically significant (CnonT+PT $\leq$ CnonT+CT; $p=0.009$), but this finding has to be regarded in the context of the unusually small variation of the triplicate IgA measurements found in the cocultures of this patient (Table 4.5). Perhaps he should be regarded as having solely a pure B cell defect for in vitro IgA production.

Table 4.5: Patients with an intrinsic B cell defect for in vitro IgA production and additional diminished T helper function (B+Th).

PATIENTS	IN VITRO IgA PRODUCTION (ng/ml)						
	CnonT+CT	CnonT+CT*	CnonT+PT	CnonT+PT*	PnonT+PT	PnonT+PT*	PnonT+CT
Sporadic							
PdG	1563 ± 24	4649 ± 673	1239 ± 88	1223 ± 34	10	<10	54 ± 7
ED[a]	689 ± 79	134 ± 22	65 ± 12	71 ± 12	35 ± 8	<10	110 ± 35
WL[a]	1270 ± 96	1718 ± 63	345 ± 80	155 ± 47	11 ± 1	47 ± 13	37 ± 16
DG[a]	952 ± 125	554 ± 85	220 ± 28	162 ± 19	<10	<10	34 ± 17
DJ[a]	548 ± 33	121 ± 60	285 ± 68	161 ± 39	<10	<10	28 ± 2
Familial							
MJ	2061 ± 217	1246 ± 138	1100 ± 245	1347 ± 120	74 ± 19	135 ± 23	90 ± 9
TJ	2061 ± 217	1246 ± 138	939 ± 241	731 ± 142	37 ± 14	43 ± 5	66 ± 17
MD	1627 ± 184	1692 ± 296	511 ± 108	399 ± 107	<10	<10	153 ± 18
TM[a]	788 ± 34	1040 ± 41	506 ± 81	519 ± 77	21 ± 6	<10	60 ± 9

C = control. P = patient. nonT = non-T cells.
T = T cells. T* = 3000 rad irradiated T cells.
[a] = diminished T helper function also for in vitro IgM production.

4.4.2 In vitro IgM production

In vitro IgM production in healthy controls showed a considerable inter-individual variation: ranging from 1319 to 17467 (mean 8655) ng IgM/ml in cultures of unseparated PBMC, and from 683 to 17243 (mean 6869) ng IgM/ml in cultures of recombined control non-T and T cells. In vitro IgM production in our patients was within the range of the controls (data not shown), except in patients TjJ, RvdB, DG and MB. Only the latter patient also had low serum IgM levels (around 40 IU/ml), serum IgM levels in the other three were normal compared to age and sex specific reference values.

Excessive T suppressor function for in vitro IgM production was not found in our patients. In 5 children (patients ED, WL, DG, DJ and TM) diminished T helper function for in vitro IgM production (and in vitro IgA production) could be established.

4.4.3 Clinical manifestations

The clinical manifestations in our patients recorded during the follow-up period and the pathogenic mechanism for defective in vitro IgA production concluded from the cell culture experiments are briefly summarized in Table 4.6.

The children with pure IgA B cell defects mainly suffered from recurrent respiratory tract infections (bronchitis, otitis). They never got serious illnesses. Three patients demonstrated a B cell defect in combination with IgA-specific excessive T suppressor function (Table 4.4). The two "sporadic" patients had only mild infections; one of them also had an allergic rhinitis despite IgE deficiency and developed diabetes mellitus later on. The girl of the "familial" group suffered from coeliac disease. The patients with a B cell defect in combination with IgA-specific diminished T helper function (Table 4.5) had only mild clinical symptoms. One boy suffered from bloody stools; a diagnosis was never made and the problem disappeared spontaneously.

An IgA B cell defect combined with a diminished T helper function for both in vitro IgA and IgM production was demonstrated in five children (Table 4.5). Four of them were "sporadic" cases. WL developed Crohn's disease after puberty. ED had recurrent urinary tract infections, but he had a vesico-ureteric reflux. Two patients have developed more severe infections, not occurring in the other IgA deficient patients: DJ was treated for osteomyelitis and DG had several episodes of lymphadenitis requiring surgical treatment.

In four children in vitro IgM production by PBMC was below the range of controls. Patient DG with recurrent lymphadenitis has already been mentioned above. Patient TjJ suffered from bloody stools and developed Crohn's disease at the age of seventeen. Patient MB had severe recurrent respiratory tract infections requiring long-term prophylactic antibiotical therapy. Patient RvdB had recurrent otitis media during the whole follow-up period of 10 years.

Table 4 6 Clinical manifestations and pathogenic mechanisms concluded from the in vitro experiments in the patients

PATIENTS	Pathogenic mechanisms	age	sex	CLINICAL MANIFESTATIONS RRTI	AD	Other
Sporadic						
SvB	–	16	M		+	
BK	–	12	F	+		
BS	B	12	F		+	
TjJ[a]	B	13	M	+		Bloody stools
MB[a]	B	9	M	+		
PM	B	18	M			Ring-chromosome 22
MvdB	B	11	M	+		
RvdB[a]	B+Ts	6	M	+		
IA	B+Ts	10	F	+	+	Diabetes mellitus
PdG	B+Th	13	M	+		
ED	B+Th[b]	3	M	+		Urinary tract infections
WL	B+Th[b]	21	M		+	Crohn's disease
DG[a]	B+Th[b]	10	F	+		Lymphadenitis
DJ	B+Th[b]	10	M	+	+	Osteomyelitis
Familial						
RF	–	7	M	+		Arthralgia
DF	–	5	M	+		
MM	B	9	M	+		Coeliac disease in early
TM	B+Th[b]	5	M	+		childhood
MJ	B+Th	15	M			Bloody stools
TJ	B+Th	13	M			
MD	B+Th	11	F	+	+	
PD	B+Ts	9	F			Coeliac disease

RRTI = recurrent respiratory tract infections
AD = atopic disease
B = diminished B cell function
Ts = excessive T suppressor function
Th = diminished T helper function
[a] = in vitro IgM production below the range of healthy controls
[b] = diminished T helper function also for in vitro IgM production

4.5 DISCUSSION

In all children investigated in this study except one a B cell defect for PWM-driven in vitro IgA production was found. Pure B cell defects were only found in a minority of our patients. The regulation of B cell differentiation to IgA-secreting plasma cells is not fully understood (19). It is known that this process is profoundly influenced by T cells and T cell-derived cytokines (19,20). In the present study coculture experiments revealed regulatory T cell disturbances in addition to defective IgA B cell function in 12 out of 22 children with selective IgA deficiency. Pure T cell defects did not occur. There were no obvious differences between "sporadic" and "familial" cases.

A B cell defect in combination with IgA-specific excessive T suppressor function was found in 3 children. Excessive T suppressor function has been reported previously, but only in a few patients it was concluded to be exclusively responsible for IgA deficiency (10). In most other reported cases excessive T suppressor function either was not IgA-specific and/or combined with B cell defects (9-12). Studies by Lynch and co-workers using murine plasmacytomas as monoclonal B cell models revealed the existence of IgA-specific T suppressor cells that acted directly on B cells (21). The involvement of regulatory mechanisms exercised by T suppressor cells on expression and function of immunoglobulin mRNA in IgA B cells has been demonstrated (22). Recently we described two families in which IgA deficient mothers with circulating anti-IgA antibodies each had two children with selective IgA deficiency. In vitro experiments similar to those in the present study revealed an IgA B cell defect and IgA-specific excessive T suppressor function in all four children (14). Considering these findings it is possible that the IgA-specific excessive T suppressor function found in three of the patients in the present study indeed contributed to the pathogenesis of their selective IgA deficiency.

An IgA B cell defect in combination with an IgA-specific diminished T helper function was found in 4 patients. To our knowledge the combination of defective B cell function with IgA-specific diminished T helper function has not been reported before in selective IgA deficiency.

The reliability of T cell dysfunction as a pathogenic mechanism would be strengthened by demonstrating identical findings within one family. In family J the same pathogenic mechanism was established in both family members. In family M only one of the children (TM) had co-existence of diminished T helper function. In family D conflicting data were found: MD showed co-existence of IgA-specific diminished T helper function, whereas her sister PD demonstrated co-existence of IgA-specific excessive T suppressor function. So it remained questionable whether T cell abnormalities (found in connection with defective IgA B cell function) represented a primary cause or became manifest later on in life.

The clinical picture of the patients with pure B cell defects and those with B cell defects and IgA-specific T cell regulatory disturbances was comparable: atopic disorders and mild respiratory tract infections.
In none of our patients excessive T suppressor function for in vitro IgM production could be established, while in several patients diminished T helper function was found for both in vitro IgA and IgM production. It has to be noted that most children with complaints other than recurrent respiratory tract infections or atopy also had disturbances in in vitro IgM production. Diminished in vitro IgM production despite normal serum IgM levels has been reported previously in patients with selective IgA deficiency (13,14,23). Decreased primary immune responses have been demonstrated in IgA deficient patients: in spite of normal and even elevated serum IgM and IgG levels there was a significantly lower response of the IgM and IgG anti-haemocyanin antibodies (5). One of our patients (WL) was investigated with haemocyanin too and showed a decreased IgM response whereas his IgG response was normal.

It has to be considered that a certain category of individuals with selective IgA deficiency in fact has a more severe immunodeficiency in which immune responses in other immunoglobulin classes than IgA are affected. In some patients with selective IgA deficiency development of common variable immunodeficiency (CVID) has been documented (15,24). The immunologic dysregulations implicated in the pathogenesis of CVID are heterogeneous and resemble those in selective IgA deficiency: intrinsic B cell defects and regulatory T cell disturbances (24). Recently it was demonstrated that individuals with IgA deficiency and CVID share polymorphisms of MHC class III genes, suggesting that these disorders indeed are related (25). Both are known for their association with the development of auto-immune diseases. One of the

MHC supratypes shared in common is strongly associated with antibody-mediated autoimmune diseases, although the mechanism is still unknown (26). The heterogeneity of clinical manifestations in patients with selective IgA deficiency possibly is based on the fact that some of them are shifting towards the development of CVID. This hypothesis is supported by the disturbances in in vitro IgM production found in some of our patients.

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Chapter 5

FAMILIAL SELECTIVE IgA DEFICIENCY WITH CIRCULATING ANTI-IgA ANTIBODIES

A distinct group of patients?

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5.1 ABSTRACT

Two families were investigated in which the mothers had selective IgA deficiency and circulating class-specific anti-IgA antibodies. Both gave birth to two children who were found to be IgA deficient. Three of these children developed anti-IgA antibodies before puberty. In vitro immunoglobulin production studies performed in the children of both families revealed an IgA B cell defect combined with IgA-specific excessive T suppressor function in all four. The mechanisms by which transplacental passage of maternal anti-IgA antibodies could have interfered with the developing IgA system in the offspring are discussed.

5.2 INTRODUCTION

Selective IgA deficiency is considered to be the most common primary immunodeficiency. Although most cases of IgA deficiency are "sporadic", the occurrence of "familial" selective IgA deficiency with various patterns of inheritance has been described (1-3).

The incidence of anti-IgA antibodies in patients with selective IgA deficiency has been reported in percentages up to 40% (1). The reason why a substantial number of selective IgA deficient patients develop anti-IgA antibodies and others do not is unknown. A relationship with previous administration of gamma-globulin or blood transfusion usually cannot be established when studying large groups of IgA deficient patients with anti-IgA antibodies, but is well known in individual cases. Presence of anti-IgA antibodies may result in hazardous reactions after transfusion of IgA-containing products (4-6). The role of anti-IgA antibodies in pathogenesis and/or maintenance of selective IgA deficiency is not clear. The possibility of induction of selective IgA deficiency by transplacental passage of maternal anti-IgA antibodies has been suggested (7-8).

Investigations on the cellular basis of selective IgA deficiency reported previously showed varying results. The majority suggest a developmental defect in IgA B cell differentiation, but disturbances in regulatory T cell functions for in vitro IgA production have also been demonstrated (9-12).

In this study we report our findings in two families in which IgA deficient mothers with class-specific anti-IgA antibodies gave birth to children who were also found to be IgA deficient. One family has been reported earlier (7), and additional follow-up is presented here. The cellular basis of selective IgA deficiency was investigated in the children of both families by means of in vitro immunoglobulin production experiments.

5.3 PATIENTS AND METHODS

5.3.1 Patients

In each family there were two children and a mother with selective IgA deficiency. Selective IgA deficiency was defined according to Hong and Ammann (13): serum IgA level: <50 mg/liter, normal serum IgG and IgM levels, and no or at most mild abnormalities of T cell function. Both fathers had normal serum IgA levels. There were no other children in the families.

Family T has been described extensively in a previous report (7). The mother (TW) was known in our hospital with selective IgA deficiency and anti-IgA antibodies. She gave birth to two children. Anti-IgA antibodies obviously had been transported across the placenta as they could be demonstrated in the cord serum and in the serum of the children after the first week of life (7). The elder boy (NT) remained IgA deficient and developed anti-IgA antibodies himself at the age of 3.5 years. His younger brother (YT) initially developed normal serum IgA levels in his first year of life (7), but was found to be IgA deficient at the age of five when he was reexamined. Anti-IgA antibodies could not be detected in his case as yet. Secretory IgA was measured in saliva and was not detectable (<2 mg/liter) in the mother and in NT. In the case of YT a level of 30 mg/liter of secretory IgA was found in saliva at the age of one. Secretory IgA was no longer detectable at the age of five.

The proband in Family L. was a 7-year-old girl (WL) referred to our department because of recurrent respiratory tract infections. She was found to be IgA deficient and anti-IgA antibodies were present. Family studies revealed a younger sister (AL) with selective IgA deficiency who developed anti-IgA antibodies 5 years later at the age of nine. Their mother (ML) was IgA deficient too, and anti-IgA antibodies were found to be present. Secretory IgA was not detectable in saliva in all three. Immunoglobulin studies during pregnancy and in the first years of life of the children from this family are not available.

5.3.2 Control

The control person used for the coculture experiments reported in this study was a healthy 30-year-old laboratory assistant with normal serum immunoglobulin levels and intact in vitro Ig production in previous pilot experiments.

5.3.3 Serum Immunoglobulin measurements

IgG, IgA, and IgM levels were determined by laser nephelometry using a DISC 120 Nephelometer (Hyland, Nivelles, Belgium). Serum IgA levels below the detection limit of the applied nephelometric technique (50 mg/liter) were further quantitated with a solid-phase radioimmunoassay (14). Class-specific anti-IgA antibodies were determined by a titration radioimmunoassay as described previously (7,14). IgG subclass levels were determined by radial immunodiffusion and calibrated against reference serum. The reference serum contained 6.2 g/liter IgG_1, 2.4 g/liter IgG_2, 0.64 g/liter IgG_3 and 0.46 g/liter IgG_4. IgG subclass levels were expressed as a percentage of the reference serum. Levels below 10% of the standard serum were considered as an indication for IgG subclass deficiency. Serum IgD levels were determined by ELISA and IgE levels by a radioimmunoassay. The lowest limit of detection was 1 IU/ml for IgD and 2 IU/ml for IgE. Undetectable values were considered as an indication for IgD or IgE deficiency.

5.3.4 Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from defibrinated venous blood of patients and the control person by Ficoll-Isopaque density gradient centrifugation. After two wash steps erythrocytes were lysed with ammonium chloride solution. Separation into T and non-T cell fractions was performed by a rosetting technique with 2-aminoethylisothiouronium bromide hydrobromide-treated fresh sheep red blood cells (AET-SRBC). The T and non-T cell fractions thus obtained were resuspended in a cell culture medium containing 25% fetal calf serum (Seralab, Crawley Down, England) in RPMI 1640 (GIBCO Europe, Paisley, Scotland) with the addition of 2.0 mmol/liter glutamine, 100,000 U/liter penicillin and 100 mg/liter streptomycin.

Investigations on cell surface markers were performed using fluorescence microscopy. Surface Ig^+ cells were stained with rhodamine-labeled polyclonal goat $F(ab')_2$ anti-human total immunoglobulins antiserum (Kallestad, Austin, TX). $CD5^+$ T cells were stained in an indirect method using Leu-1 monoclonal antiserum (Becton Dickinson, Sunnyvale, CA) and fluorescein-labeled goat anti-mouse immunoglobulin antiserum. $CD4^+$ and $CD8^+$ T cells were stained in a similar way using OKT4 and OKT8 monoclonal antisera (Coulter Immunology, Hialeah, FL).

5.3.5 Cell cultures

Cultures always consisted of 3×10^5 cells in 1 ml of culture medium and were incubated in round-bottomed tubes in triplicate for 9 days at 37°C in a 5% CO_2, high-humidity incubator. Unseparated PBMC were cultured in order to relate the number of cytoplasmic IgA-containing cells to the amount of IgA secreted in the culture supernatant. In two experiments T and non-T cell fractions of the children of each family and the control person were either autologously recombined or cocultured with their respective counterpart cell fractions. Cocultures consisted of 1.5×10^5 non-T cells and 1.5×10^5 T cells, without correction for the number of monocytes. Each coculture was also performed with irradiated T cells (2000, 2500 or 3000 rad from a cesium-sourced Gammacell 1000B blood cell irradiator, Isomedix Inc, Parsippany, NJ) to eliminate T suppressor function (15). Cells were cultured with and without stimulation by pokeweed mitogen (PWM, GIBCO Laboratories, Grand Island, New York) in a final concentration of 12.5 ug/ml of culture medium. Supernatants were collected and stored at -20°C until determination of immunoglobulin content.

5.3.6 Immunoglobulin assays in the culture supernatants

Supernatant IgA and IgM contents were measured in duplicate after 9 days of culture using a class-specific enzyme-linked double-antibody immunosorbent assay (ELISA). Briefly, coating was performed with rabbit anti-human IgA or IgM gammaglobulin fraction (DAKO, Glostrup, Denmark) and horseradish peroxidase-labeled rabbit anti-human IgA or IgM (DAKO) was used as second antibody. The chromogene was H_2O_2 with ortho-phenylenediamine-di-HCl. Values were expressed in terms of

nanograms of Ig per milliliter. The lowest limit of detection was 10 ng/ml for IgA and 20 ng/ml for IgM. The variation of the duplicate ELISA immunoglobulin measurements ranged from 3 to 6% for both IgA and IgM. The variation in immunoglobulin production within the triplicate set of cultures ranged from 15 to 30% for both IgA and IgM.

5.3.7 Detection of cytoplasmic IgA

After removal of the supernatant, the cells of the triplicate set of culture tubes containing unseparated PBMC were combined. After washing and fixation with 0.5% formaldehyde, cytospin slides were made, and cytoplasmic IgA was stained with fluorescein-labeled goat $F(ab')_2$ anti-human IgA (Kallestad). IgA-containing cells were expressed as a percentage of the total cell amount (at least 1000 cells were counted).

5.3.8 Calculations

One-sided t tests modified according to Satterthwaite were used for comparison of cocultures within one patient (TTEST-SAS procedure). The Bonferroni adjustment was applied to determine significance levels for analysis involving multiple comparisons. This implied a significance level of $p = 0.0125$ for IgA (four comparisons) and $p = 0.01$ for IgM (five comparisons).

5.4 RESULTS

Immunological parameters and clinical manifestations of the IgA deficient members of both families are presented in Table 5.1. In all patients except YT, a concomitant IgG_4-IgE deficiency could be demonstrated. Both mothers both showed elevated serum IgM levels. Surface IgA^+-cells were absent in all patients.

Table 5.1: Immunological parameters and clinical manifestations of the IgA deficient members of both families.

	Family T			Family L		
patients	TW	NT	YT	ML	WL	AL
age in years	32	10	7	35	12	10
serum immunoglobulins						
IgG (IU/ml)	113	120	140	200	99	110
IgA (mg/liter)	nd	nd	4.3	nd	nd	nd
IgM (IU/ml)	318	81	197	448	108	122
IgD (IU/ml)	<1	<1	120	8	<1	<1
IgE (IU/ml)	<2	<2	55	<2	<2	<2
IgG_1 (%)[a]	150	160	160	197	105	95
IgG_2 (%)	50	20	92	152	26	50
IgG_3 (%)	92	100	98	138	78	112
IgG_4 (%)	<10	<10	28	<10	<10	<10
anti-IgA antibodies[b]	5	1.1	nd	4.4	25	0.4
Cell markers						
surface Ig^+ cells (%)	10	13	18	14	20	8
surface IgA^+ cells (%)	nd	nd	nd	nd	nd	nd
E-rosetting cells (%)	64	63	68	47	48	55
Symptoms	GI	OME	none	none	RRTI	none

NOTE : nd = not detectable. GI = gastro-intestinal: diarrhoea, giardiasis, follicular hyperplasia. OME = recurrent otitis media with effusion. RRTI = recurrent respiratory tract infections, bronchiectasies, impaired lung function.

[a] IgG-subclass levels are expressed as a percentage of reference serum (H00-020, The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service).

[b] The titers of neutralizing anti-IgA antibodies are expressed in mg IgA/liter added (14).

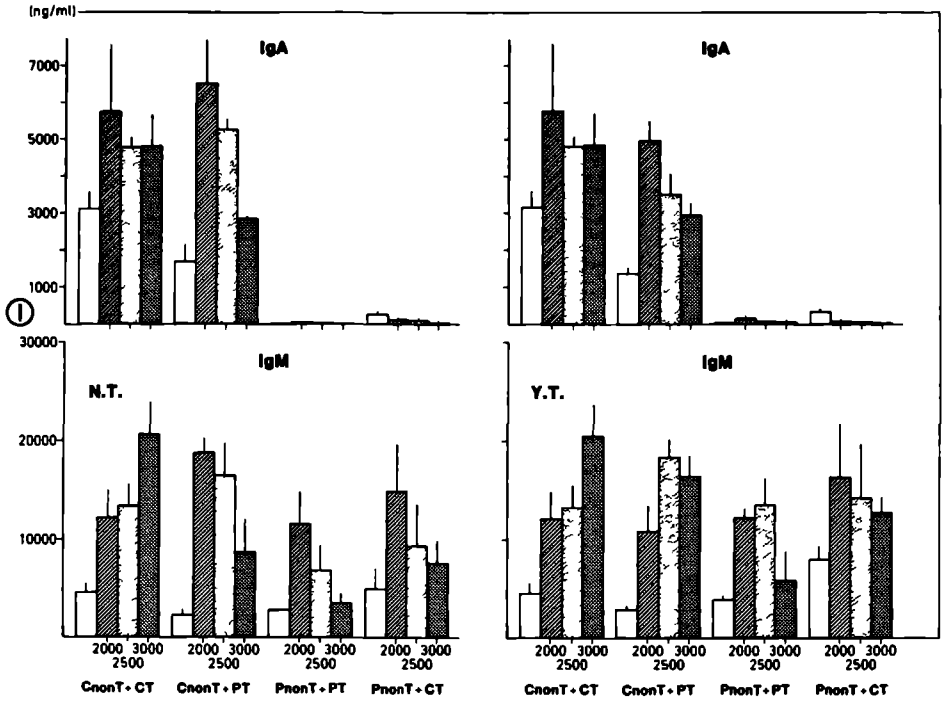
In vitro IgA production by unseparated PBMC and the number of cells staining for cytoplasmic IgA after PWM-stimulated cell culturing are presented in Table 5.2. Compared to healthy controls, in vitro IgA production and the number of cytoplasmic IgA containing cells were clearly lower in the patients with selective IgA deficiency.

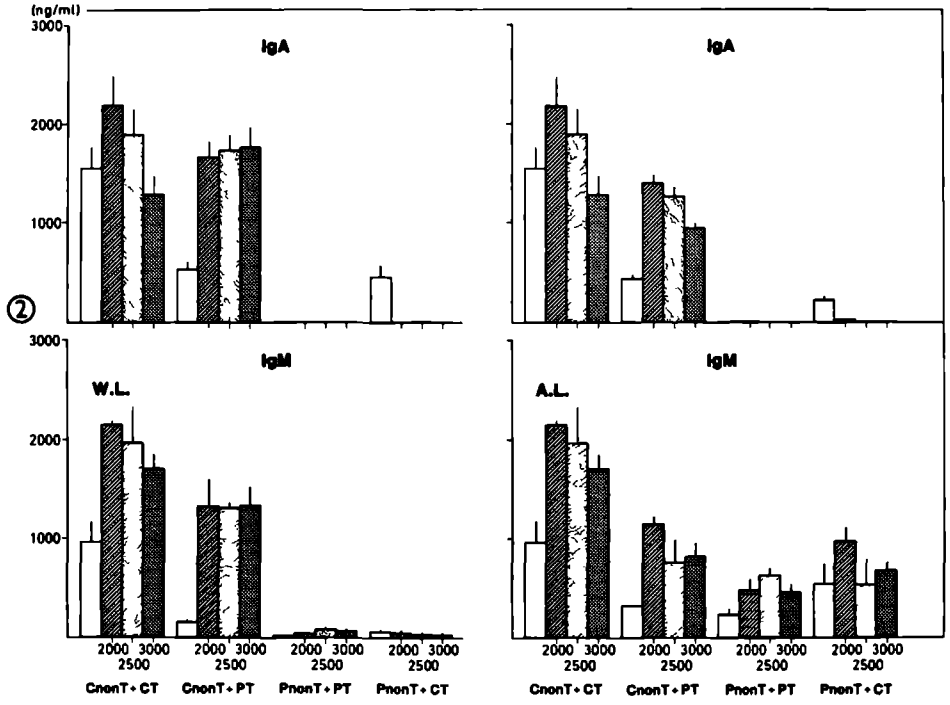
Table 5.2: PWM-stimulated culture of unseparated PBMC of both families with selective IgA deficiency.

	IgA-containing plasmacells[a]	In vitro IgA production[b]
Family T		
NT	<0.1	<10
YT	0.3	<10
Family L		
WL	<0.1	<10
AL	<0.1	50
healthy controls (n=11)	2.0-10.0	698-4884

[a] The number of cytoplasmic IgA-containing cells is expressed as a percentage of the total cell amount on the cytospin slide.
[b] In vitro IgA production is expressed in terms of nanograms of IgA/ml of culture supernatant.

In vitro Ig production by NT and YT in the cocultures is presented in Figure 5.1. WL and AL were investigated in a separate experiment using the same control person (Figure 5.2). Although the applied culture techniques were identical to those in the first experiment, the amounts of IgA and especially IgM measured in the culture supernatants of the second experiment turned out to be much lower.


(ng/ml)
IgA
IgA
7000
5000
3000
1000
①
30000
IgM
N.T.
IgM
Y.T.
20000
10000
2000 3000 2500
CnonT+CT
CnonT+PT
PnonT+PT
PnonT+CT


(ng/ml)
3000
IgA
IgA
2000
1000
②
3000
IgM
W.L.
IgM
A.L.
2000
1000
2000 3000 2500
CnonT+CT
CnonT+PT
PnonT+PT
PnonT+CT

Figures 5.1 and 5.2: In vitro IgA and IgM production by lymphocytes from a healthy control person and four children with "familial" IgA deficiency. Cocultures were performed after recombination of equal numbers of non-T and T cells ($1.5x10^5$). C = control; P = patient; open bars represent cocultures with nonirradiated T cells; striped bars cocultures with irradiated T cells (2000, 2500 and 3000 rad, respectively). All values are expressed in terms of nanograms of immunoglobulin/ml and shown as the calculated mean of the triplicate set of each recombination. The line on top of the bars represents the standard deviation.

IgA production by recombined patient non-T and T cells was absent or very low. Recombination of patient non-T cells with irradiated patient T cells revealed similar results. Coculture of patient non-T cells with control T cells resulted in detectable amounts of IgA production (Figures 5.1 and 5.2). This was probably caused by residual non-T cells in the control T cell fraction (Table 5.3). This supposition is proven by the fact that if control T cells were irradiated prior to culture to eliminate immunoglobulin production by residual control non-T cells, IgA production again was absent or very low and comparable to that of recombined patient non-T and T cells (Figures 5.1 and 5.2). These findings evidence the existence of an intrinsic B cell defect in all children.

Coculture of control non-T cells and patient T cells showed a significantly ($p \leq 0.0125$) lowered in vitro IgA production compared to recombination of control non-T and T cells (Table 5.4). However, if patient T cells were irradiated prior to culture, in vitro IgA production in these cocultures was comparable to that of recombined control non-T and T cells (Figures 5.1 and 5.2). These findings indicate coexistence of radio-sensitive excessive T suppressor function besides the intrinsic B cell defect in all children.

In vitro IgM production was measured simultaneously in all cocultures. In all children except WL, in vitro IgM production by recombined patient non-T and T cells and by cocultured patient non-T and control T cells was comparable to that of recombined control non-T and T cells, indicating normal in vitro IgM B cell function (Figures 5.1 and 5.2). In the case of WL, IgM was undetectable in the culture supernatant after culturing recombined patient non-T and T cells. If her T cells were irradiated prior to culture, in vitro IgM production could be detected, but only in low amounts compared to the control person (Figure 5.2).

Table 5.3: Cell surface markers before and after separation of PBMC in non-T and T cell fractions.

		Family T			Family L		
		NT	YT	control	WL	AL	control
BEFORE SEPARATION							
surface Ig^+-cells	(%)	13	18	13	20	8	11
$CD5^+$-cells	(%)	54	54	64	49	45	63
$CD4^+$-cells	(%)	47	38	39	40	34	–
$CD8^+$-cells	(%)	7	10	25	9	9	–
AFTER SEPARATION							
non-T cells							
surface Ig^+-cells	(%)	32	43	28	45	22	36
$CD5^+$-cells	(%)	<1	2	1	<1	<1	<1
T cells							
surface Ig^+-cells	(%)	5	4	2	4	1	2
$CD5^+$-cells	(%)	80	77	84	79	84	81
$CD4^+$-cells	(%)	66	57	63	69	65	52
$CD8^+$-cells	(%)	10	14	25	10	11	28

The difference observed in all patients between in vitro IgM production of recombined control non-T and T cells and cocultured control non-T and patient T cells was not statistically significant (Table 5.4). This indicates that there was no gross abnormality in the patients' T cell regulatory functions for in vitro IgM production. Regarding the cultures containing irradiated patient T cells, an elevation of in vitro IgM production was observed on several occasions (Figures 5.1 and 5.2), although not always statistically significant (Table 5.4). A comparable elevation of in vitro IgM production was noted if control non-T cells were recombined with irradiated control T cells (Figures 5.1 and 5.2).

Table 5.4: Analysis of coculture results using one-sided t-tests modified according to Satterthwaite (TTEST-SAS procedure).

COMPARISONS			PATIENTS NT	YT	WL	AL
IgA						
PnonT + CT	≤	CnonT + CT	+	+	+	+
CnonT + PT	≤	CnonT + CT	+	+	+	+
CnonT + PT*	≤	CnonT + CT	– – –	– – –	– – –	– – –
CnonT + PT*	≥	CnonT + PT	+ + –	+ + +	+ + +	+ + +
conclusion			**B+Ts**	**B+Ts**	**B+Ts**	**B+Ts**
IgM						
PnonT + CT	≤	CnonT + CT	–	–	+	–
CnonT + PT	≤	CnonT + CT	–	–	–	–
CnonT + PT*	≤	CnonT + CT	– – –	– – –	– – –	– – –
CnonT + PT*	≥	CnonT + PT	+ + –	– + +	+ + +	+ – –
PnonT + PT*	≥	PnonT + PT	– – –	+ – –	– – –	– + –
conclusion					**B**	

NOTE:
- P = Patient
- C = Control
- PT* = irradiated patient T cells; 2000, 2500 or 3000 rad respectively
- \+ = p-value ≤ 0.0125 for IgA (Total p-value ≤ 0.05)
  p-value ≤ 0.0100 for IgM (Total p-value ≤ 0.05)
- – = not significant
- B = B cell defect: (PnonT+CT ≤ CnonT+CT : +)
- Ts = excessive T suppressor function: (CnonT+PT* ≤ CnonT+CT : –, all other comparisons : +)

5.5 DISCUSSION

All four children in the two investigated families are IgA deficient.

In Family T we were able to demonstrate transplacental passage of maternal anti-IgA antibodies (7). NT remained IgA deficient after birth. YT initially showed a rise in serum IgA levels and IgA was found present in saliva, but eventually he did not develop a mature IgA system.

We suggested the possibility of a disturbance in the IgA-specific T cell regulation, induced by maternal anti-IgA antibodies, and resulting in inhibition of IgA B cell differentiation and proliferation in the children (7). Two of the patients with selective IgA deficiency and disturbance in regulatory T cell function reported by Waldmann et al. (9) also had mothers with high titers of anti-IgA antibodies, and induction of allotype specific T suppressor cells in the progeny was suggested.

Our investigations on the cellular basis of IgA deficiency in the children presented in this paper revealed an intrinsic B cell defect for in vitro IgA production in all four. The low numbers of cytoplasmic IgA containing cells after 9 days of culture evidenced that this B cell defect was not due to a secretory blockade. Furthermore, a coexisting excessive T suppressor function was found in all children. This probably appeared to be IgA-specific, as we were not able to demonstrate a clearly significant effect upon in vitro IgM production.

It is known from several studies that administration of an anti-immunoglobulin can lead to a state of idiotype-specific suppression which, once established, can be transferred by T cells (16-18). In a sequence of studies by Lynch and co-workers (reviewed in Ref.(19)) using murine plasmacytomas as monoclonal B cell models, immunoglobulin-specific T suppression was further examined. This revealed that the target of the suppressor T cell is a B cell and not a T helper cell. It was found that the initial encounter between the B cell and the T suppressor cell is highly specific and relies on the recognition of an idiotope located in the variable region of the alpha heavy chain. In the suppressed IgA B cell inhibition of the light chain messenger RNA could be demonstrated, eventually preventing the expression of both the heavy (alpha) and the light chain polypeptides (20).

As we were able to demonstrate transplacental passage of anti-IgA antibodies in the two children of one family, and considering the possibility of anti-IgA transmission in the second family, the B cell defect and the excessive T suppressor function established in the children of both families could be the result of similar mechanisms as described above. Transplacental anti-IgA antibodies could have generated T suppressor cells in the children that, in turn, could have down-regulated IgA synthesis by an effect on IgA B cells. This hypothesis would explain the eventual failure of development of a mature IgA system in patient YT.

Three of the four children developed anti-IgA antibodies before puberty. In a longitudinal survey of 40 children with selective IgA deficiency known to our department, only in these three children could

anti-IgA antibodies be demonstrated before puberty (PCJ De Laat et al. submitted for publication). None of them underwent a transfusion with blood or other IgA-containing products that might have induced development of anti-IgA antibodies. Our findings indicate a predisposition in children from mothers with anti-IgA antibodies to develop anti-IgA antibodies before puberty. Furthermore, in these three patients and their mothers a concomitant IgG_4-IgE deficiency was found (Table 5.1). Increased frequencies of anti-IgA antibodies have been reported in IgA deficient patients with concomitant IgG subclass and/or IgE deficiency (5,6,21,22). In this respect it will be interesting to see whether YT will develop anti-IgA antibodies in the near future, as at present he has normal serum levels of IgE and IgG_4.

In conclusion, the children investigated in this study appear to form a distinct group of patients. Maturation of their IgA system seems to be influenced in the perinatal period by maternal anti-IgA antibodies. The possibility of induction of IgA-specific excessive T suppressor function that affects IgA B cell function, and finally results in selective IgA deficiency has to be considered. In contrast to all other children with selective IgA deficiency known to our department, a tendency was found for development of anti-IgA antibodies before puberty in these children.

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Chapter 6

IgA DEFICIENCY IN PATIENTS WITH JUVENILE CHRONIC ARTHRITIS TREATED WITH GOLD

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6.1 ABSTRACT

Three patients with juvenile chronic arthritis treated with aurothioglucose developed a significant fall in serum IgA levels. One of them showed persistence of IgA deficiency during 3.6 years of chrysotherapy. Serum IgA levels after cessation of chrysotherapy remained below the normal range in the second patient. In the third patient serum IgA returned to pretreatment values. There were no other side-effects, and the incidence of infections was not increased. In vitro experiments demonstrated diminished IgA B cell function in all 3 patients. At least one of the two patients who were still treated with gold at the time the experiments were performed had an also diminished IgM B cell function.

6.2 INTRODUCTION

Development of IgA deficiency is a wellknown side-effect of the slow-acting antirheumatic drugs used in the treatment of adults with rheumatoid arthritis. Associations were found with D-penicillamine (1-3), sulfasalazine (4) and gold (1-3,5). Development of IgA deficiency was also described in treatment of Wilson's disease with D-penicillamine (6,7), of epilepsy with phenytoin (8,9), and of ulcerative colitis with sulfasalazine (10-12). Development of IgA deficiency during chrysotherapy in children with juvenile chronic arthritis (JCA) has been described (13). The mechanism of drug interference with immunoglobulin (Ig) synthesis is largely unknown.

In this study we report 3 patients with JCA who developed a significant fall in serum IgA levels after treatment with aurothioglucose. The capacity for in vitro Ig production was studied in pokeweed mitogen (PWM)-driven cell cultures.

6.3 METHODS

6.3.1 Serum immunoglobulin measurements

IgG, IgA and IgM levels were determined by laser-nephelometry using a DISC 120 Nephelometer (Hyland, Nivelles, Belgium), and all values were calibrated against WHO standard serum No. 67/97. Serum IgA levels below the detection limit of the applied nephelometric technique (2 IU/ml) and, if present, the amount of anti-IgA antibodies were quantitated with a solid-phase radioimmunoassay (14). Saliva IgA levels were determined with a radioimmunoassay.

6.3.2 Cell culture experiments

Cell preparation and cell cultures were performed as described in Chapter 3. Briefly, peripheral blood mononuclear cells (PBMC) from patients and controls were isolated by density-gradient centrifugation and separated into T and non-T cell fractions. T and non-T cells from controls and patients were either autologously recombined or cocultured with respective counterpart cells in triplicate PWM-driven cell cultures. Each cell culture was also performed with irradiated T cells (3000 rads)

to eliminate T suppressor function (16). IgA and IgM concentrations in the culture supernatants were measured by ELISA and expressed as nanograms of Ig per ml supernatant.

6.3.3 Controls

The controls used in the coculture experiments were 2 healthy laboratory workers with normal serum Ig levels and intact in vitro Ig production.

6.3.4 Calculations

A one-sided t-test modified according to Satterthwaite was used for comparison of certain cell cultures within one patient-control combination (TTEST-SAS procedure). The Bonferroni adjustment was applied to determine significance levels for analysis involving multiple comparisons. This implied a significance level of $p=0.0125$ for IgA (four comparisons), and $p=0.0100$ for IgM (five comparisons). According to the Bonferroni principle (the sum of multiple tests) the total level of significance of a pathogenic mechanism concluded from the coculture results then will be $p=0.05$. The conditions to distinguish defective B cell function, excessive T suppressor function and diminished T helper function were set as follows (see also Chapter 3):

Diminished B cell function was concluded if Ig production by cocultures of patient non-T cells + control T cells (PnonT+CT) was significantly lower than Ig production by recombined control non-T + T cells (CnonT+CT).

Excessive T suppressor function was only concluded if the following conditions were all three fulfilled: Ig production by cocultures of CnonT + patient T cells (CnonT+PT) must be significantly lower than Ig production by recombined CnonT+CT; Ig production by cocultures of CnonT + irradiated PT (CnonT+PT*) cells must not be significantly different from Ig production by CnonT+CT cells; and Ig production by cell cultures containing irradiated PT cells must be significantly higher than Ig production by cell cultures containing non-irradiated PT cells.

Diminished T helper function was only concluded if the following conditions were all three fulfilled: Ig production by cocultures of CnonT + patient T cells (CnonT+PT) must be significantly lower than Ig production by recombined CnonT+CT; Ig production by cocultures of

CnonT + irradiated PT (CnonT+PT*) cells must be significantly lower than Ig production by CnonT+CT cells; and Ig production by cell cultures containing irradiated PT cells must not be significantly higher than Ig production by cell cultures containing non-irradiated PT cells.

6.4 RESULTS

6.4.1 Clinical and Immunological findings

Some data of the patients at the time of the in vitro experiments and the longitudinal serum Ig levels are shown in Table 6.1 and Figure 6.1.

Table 6.1: Data of the three patients at the time of the cell culture experiments.

	PATIENTS		
	1	2	3
Age (yrs)	6.1	17.7	15.6
Sex	F	F	M
Duration of gold therapy (mths)	12	57*	9
Serum Ig levels			
IgA (IU/ml)	0.1	27	36
IgG (IU/ml)	149	139	155
IgM (IU/ml)	164	85	121
HLA pattern	A2 A9 B40 B15 Bw6 Bw60 Bw62 DR2 DR3 DRw52 DQw1 DQw2	A1 B7 B8 Bw6 DR3 DR5	A2 A9 B7 B18 Bw6 DR1 DRw6 DRw52 DQw1

* = cultures were performed 24 months after cessation of chrysotherapy.

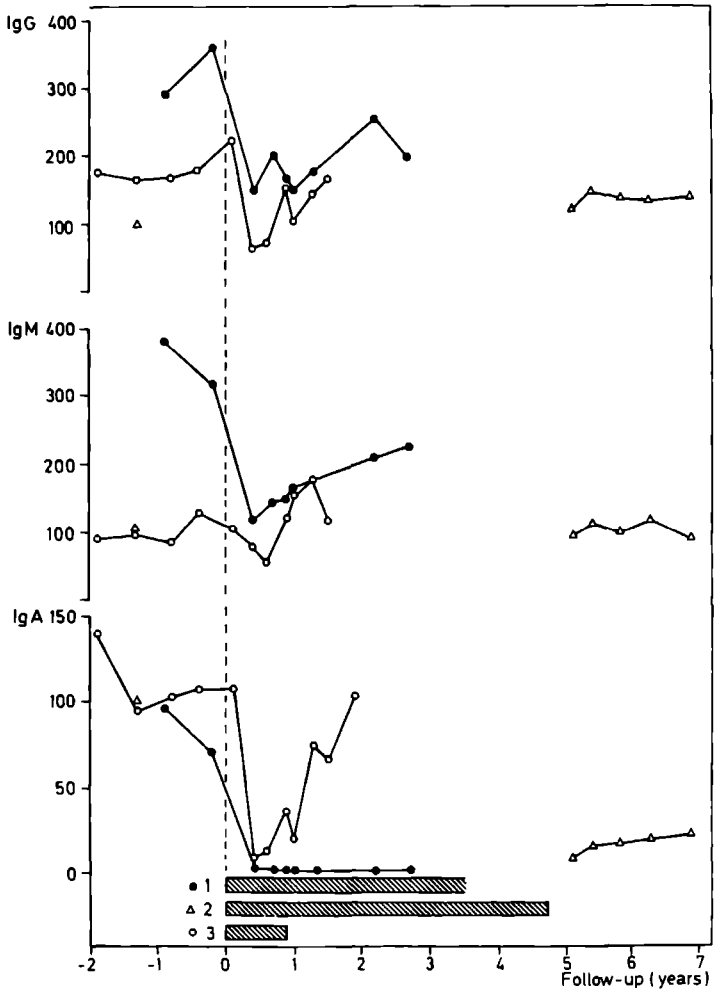

Figure 6.1: *Longitudinal serum IgG, IgM and IgA levels (in IU/ml) in 3 patients with JCA before, during and after treatment with aurothioglucose.*
T_0 = start gold therapy.

Patient 1 is a girl with seronegative polyarticular JCA since the age of 4 years. Gold therapy was started at the age of 5 years, given as weekly intramuscular injections of aurothioglucose (1 mg/kg/dose) during 20 weeks while therapy with non-steroidal anti-inflammatory drugs was continued. Depending on the clinical improvement, the gold injections were then gradually reduced to every 2, 3, 4, and then every 6 weeks. Serum Ig levels before and during treatment with aurothioglucose are shown in Figure 6.1. After 5 months of chrysotherapy the serum IgA level was found to be very low (0.1 IU/ml) and IgA was undetectable in saliva. This patient is still on gold therapy and serum IgA remained below 0.1 IU/ml (2 mg/l). Anti-IgA antibodies could not be detected. The incidence of infections since the development of selective IgA deficiency was not increased. Other aurothioglucose-induced side-effects did not occur in the past 3.6 years of chrysotherapy.

Patient 2 is a girl with seronegative polyarticular JCA since the age of 9 years. Treatment with aurothioglucose was started at the age of 10 years, and discontinued after 57 months because of clinical improvement. She had normal adult values of serum IgA, IgG and IgM levels before treatment. Serum Ig levels during chrysotherapy were not available. There were no obvious aurothioglucose-induced side-effects, and the incidence of infections during chrysotherapy or thereafter was not increased. Serum Ig levels after cessation of chrysotherapy are shown in Figure 6.1. Three months after the gold injections were stopped the serum IgA level was found to be 7 IU/ml. Later on, IgA levels increased gradually but remained below -2SD for the age-specific normal values.

Patient 3 is a boy with systemic-onset JCA since the age of 12 years. Because of severe polyarticular arthritis aurothioglucose injections were started at the age of 14 years when systemic symptoms such as fever and rash were already absent since a longer period. Gold therapy was discontinued after 9 months because of insufficient clinical effect. Serum Ig levels before, during and after treatment with aurothioglucose are shown in Figure 6.1. After 4 months of chrysotherapy the serum IgA level was found to be 9 IU/ml, gradually increasing to 36 IU/ml just before the gold injections were stopped. Serum IgA levels returned to pretreatment values after cessation of chrysotherapy (Figure 6.1). There were no other aurothioglucose-induced side-effects, and the incidence of infections was not increased.

6.4.2 In vitro experiments

In vitro IgA production (Table 6.2):

In all 3 patients a diminished B cell function was established for in vitro IgA production. In patients 2 and 3 cocultures of patient non-T + control T cells (PnonT+CT) produced significantly lower amounts of IgA compared to the IgA production by recombined control non-T + T cells (CnonT+CT). In patient 1 the IgA production resulting from co-culturing PnonT+CT cells probably was due to residual B cells in the CT cell fraction. This was proven by the fact that IgA production could no longer be detected if CT cells were irradiated prior to coculture (PnonT+CT*), so preventing Ig production by residual B cells.

In all 3 patients in vitro IgA production by cocultured control non-T + patient T cells (CnonT+PT) was not significantly lower than IgA production by recombined CnonT+CT cells, indicating that there was no gross abnormality in patients' T cell regulatory functions for in vitro IgA production.

In vitro IgM production (Table 6.2):

In patients 1 and 3 cocultures of PnonT+CT cells resulted in significantly decreased amounts of IgM production compared to recombined CnonT+CT cells. This could indicate a diminished capacity of these patients' B cells for in vitro IgM production, although at the time the cell cultures were performed their serum IgM levels were normal (Table 6.1). In a previous experiment in vitro IgM production by recombined non-T and T cells in healthy controls showed considerable inter-individual variation ranging from 683-17,243 (mean 6869) ng IgM per ml (Chapter 4). Regarding the cocultures containing patient non-T cells in the present experiments only in patient 1, but not in patient 3 in vitro IgM production was certainly lowered (Table 6.2). In patient 2 in vitro IgM B cell function was normal.

In vitro IgM production by cocultured CnonT+PT cells was not significantly lower than IgM production by recombined CnonT+CT cells in patients 1 and 2. In patient 3 this comparison revealed a significantly ($p < 0.01$) lower IgM production, and a substantial increase in in vitro IgM production was noted in the cultures containing irradiated T cells. This could suggest presence of excessive T suppressor function, but not all conditions set for excessive T suppressor function (see Methods section) were fulfilled.

Table 6.2: In vitro IgA and IgM production in cultures containing cells of healthy controls and/or patients expressed as nanograms of Ig per ml culture supernatant (mean ± SD).

IN VITRO IgA PRODUCTION (ng/ml)

CnonT+CT	CnonT+CT*	CnonT+PT	CnonT+PT*	PnonT+PT	PnonT+PT*	PnonT+CT	PnonT+CT*
Patient 1							
752 ± 176	965 ± 165	304 ± 18	272 ±164	<10	<10	344 ± 54	<10
Patient 2							
1158 ± 78	1729 ± 265	1253 ±153	1271 ±280	170 ± 29	485 ± 74	240 ± 53	446 ± 103
Patient 3							
2640 ± 266	2914 ± 320	1914 ±239	2860 ±407	117 ± 14	737 ±136	266 ± 59	823 ± 34

IN VITRO IgM PRODUCTION (ng/ml)

CnonT+CT	CnonT+CT*	CnonT+PT	CnonT+PT*	PnonT+PT	PnonT+PT*	PnonT+CT	PnonT+CT*
Patient 1							
4318 ± 882	8408 ± 588	1907 ± 78	5611 ±2272	46 ± 21	226 ±104	480 ±199	154 ± 37
Patient 2							
7071 ±1632	12240 ± 898	3657 ±715	9729 ± 981	2082 ±399	4917 ±780	2610 ±846	3387 ±1188
Patient 3							
3577 ± 302	8691 ±1124	1242 ±417	7750 ±1821	1492 ± 33	9027 ±521	1633 ±338	9992 ±1058

C = control P = patient nonT = non-T cells
T = T cells T* = 3000 rad irradiated T cells

6.5 DISCUSSION

The influence of aurothioglucose therapy on IgA levels was rather heterogeneous. One patient developed persistent nearly undetectable IgA levels in serum and saliva. The second patient was found to have a very low serum IgA level after 57 months of chrysotherapy. Although a gradual increase was noted, IgA levels remained below the normal values. This is in accordance with previous studies that reported persistence of low to very low serum IgA levels after treatment with slow-acting anti-rheumatic drugs (1,2,5,10,13). In the third patient serum IgA levels began to rise while he was still receiving gold injections. The clinical effect of gold therapy was poor, and probably the rise in serum IgA levels is a reflection of his increased rheumatic activity.

An association between toxic side-effects of gold compounds and certain HLA antigens (mainly B8 and DR3) has been described (17-19). Van Riel et al. suggested a possible association between lowered serum IgA concentrations and susceptibility to aurothioglucose-induced toxic effects (19). Wijnands found no differences in saliva IgA levels between toxic and non-toxic patients with rheumatoid arthritis treated with gold (20). In spite of the decrease in serum IgA levels in our small group of 3 patients, toxic effects of aurothioglucose were not observed. A common HLA pattern could not be established, and only one patient had B8 DR3. The mechanism by which aurothioglucose or other slow-acting anti-rheumatic drugs influence the IgA system remains obscure. Disturbances in Ig synthesis at the T cell level have been reported (1,5,21). Our in vitro Ig production experiments revealed a diminished IgA B cell function without evidence for concomitant disturbances of T cell regulatory functions in all 3 patients. In addition, an also diminished capacity for in vitro IgM production was established with certainty in one of the patients. The coculture studies showed diminished IgM B cell function in two patients. In one of them this was somewhat less pronounced, possibly to be explained by his increased rheumatic activity. However, in contrast with patient 2, both these patients were still on chrysotherapy at the time the cell cultures were performed. Therefore, interference of aurothioglucose with in vitro IgM B cell function too seems possible.

IgA deficiency can be the cause of increased susceptibility to infections, especially respiratory tract infections (22,23). In our 3 patients however, no increased incidence of infections was noted. In a previous study of 22 children with primary selective IgA deficiency we have found that additional disturbances in in vitro IgM production may result in more

serious clinical manifestations (Chapter 5). One of the patients in the present study (patient 1, Table 6.2) definitely had defective in vitro IgM production. However, besides JRA she had no other complaints.

In conclusion, treatment of JCA with aurothioglucose can result in decreased serum Ig levels, especially of IgA. In vitro studies in our patients revealed diminished B cell functions. Toxic side-effects of aurothioglucose were not observed and the rate of infections was not increased.

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Chapter 7

IMMUNOGLOBULIN LEVELS DURING FOLLOW-UP OF CHILDREN WITH SELECTIVE IgA DEFICIENCY.

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7.1 ABSTRACT

Longitudinal serum immunoglobulin levels were studied in 36 children with selective IgA deficiency during a median follow-up period of 5 years. Twentyfive children were "sporadic" cases, and 11 were "familial". Serum and saliva IgA levels in 23 children remained below 2 mg/l. Eight children with IgA levels above 2 mg/l showed considerable intra-individual variance in serum IgA, but remained IgA deficient. Five children at various ages developed IgA levels above 50 mg/l with detectable secretory IgA in saliva. In most of the children IgG subclass levels were found to be rather high, also at younger ages.
There were no obvious differences between "sporadic" and "familial" cases except an association between IgD deficiency and serum IgA levels below 2 mg/l, and between serum levels of IgD above 1 IU/ml and of IgA above 2 mg/l, that was found to be significant in the "sporadic" group, but not in the "familial" group.

7.2 INTRODUCTION

Selective IgA deficiency is the most frequent primary immunodeficiency (1-3). Clinical manifestations are heterogeneous, and vary from none at all to associations with auto-immune disease, recurrent infections, and atopic disease (1-4). Familial occurrence of selective IgA deficiency with various patterns of inheritance has been reported (4-5), but most patients described represent sporadic cases.

Concomitant deficiencies of one or more IgG subclasses, IgD and/or IgE have been reported in IgA deficient individuals (6-10). Serum IgA levels are not constant, and even at levels below 0.5 mg/l significant fluctuations were noted (11). Several cases of transient IgA deficiency have been found in follow-up studies (4,12). However, most data on serum immunoglobulin levels in patients with selective IgA deficiency are derived from cross-sectional studies in selected populations.

In this study we report longitudinal immunoglobulin levels in a group of 36 children during a median follow-up period of five years.

7.3 PATIENTS AND METHODS

7.3.1 Patients

IgA deficiency was defined according to Hong and Ammann (2): serum IgA level below 50 mg/l, normal serum IgG and IgM levels, and no or at most mild abnormalities of T cell function. Diagnosis was only made after the age of one year. Sibs and parents of twenty-five children had normal serum IgA levels, and these children were designated as "sporadic". The remaining eleven children represent cases of "familial" IgA deficiency. The clinical manifestations in our patients have been reported previously, and mainly consisted of mild respiratory tract infections (13).

The median age at diagnosis was 5.4 years (range 1 to 16). The median follow-up period was 5 years (range 2 to 10). Out-patient follow-up visits took place at least 2-3 times yearly, serum immunoglobulin levels were measured, and all patients were checked for development of anti-IgA antibodies. In 32 children secretory IgA was measured in saliva once or twice.

7.3.2 Methods

IgG and IgM levels in serum and saliva were measured by a single radial immunodiffusion according to Van Munster et al. (14) or by laser-nephelometry. All values were calibrated against WHO standard serum No. 67/97. Age and sex specific reference values have been determined in an earlier study (15).

IgA levels in serum and saliva (and if present the amount of class-specific anti-IgA antibodies) were measured by a titration-radioimmunoassay as described previously (16). In normal controls (n=13) the mean IgA level in saliva was 35 mg/l (SD 10, range 15-48 mg/l). IgG subclass levels were measured by radial immunodiffusion and calibrated against reference serum containing IgG, 6.2 g/l; IgG_2 2.4 g/l; IgG_3 0.64 g/l and IgG_4 0.46 g/l (HOO-020, The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). IgG_1, IgG_2 and IgG_3 levels were compared to the age related normal values described by Plebani et al. (17). Values below the third percentile (P_3) were considered as an indication of deficiency of the respective IgG subclass. Due to the very broad spread in childhood, reference values for IgG_4 are not available, and IgG_4 deficiency is difficult to define (17). In our study an arbitrary level of 0.046 g/l was chosen to delineate at least very low levels, probably indicating concomitant IgG_4 deficiency.

Serum IgD levels were measured by ELISA and serum IgE levels by radioimmunoassay. Levels below the detection limits (1 and 5 IU/ml, respectively) were considered as an indication for IgD or IgE deficiency. Serum IgE was designated as "high" if measurements were above +1 SD of the reference values provided by Kjellman (18).

7.4 RESULTS

7.4.1 Serum Immunoglobulins

IgA was detectable in very small amounts in serum of all patients except an 18-year-old girl who was the only patient with circulating class-specific anti-IgA antibodies. During the follow-up period IgA levels in 23 of our patients remained below 2 mg/l. One of them initially had IgA levels around 50 mg/l while she suffered from lymphadenitis. Within 2 months IgA levels declined to below 2 mg/l.

Considerable fluctuations in serum IgA levels were found in 8 children, but all remained IgA deficient (Figure 7.1). The relative elevations of serum IgA in these patients were not related to periods with an increased rate of infections. Associations between longitudinal serum IgA levels and longitudinal serum levels of IgG or IgM could not be established. In 5 children serum IgA levels rose above 50 mg/l, but none of them attained the lower range of age and sex specific reference values (Figure 7.2).

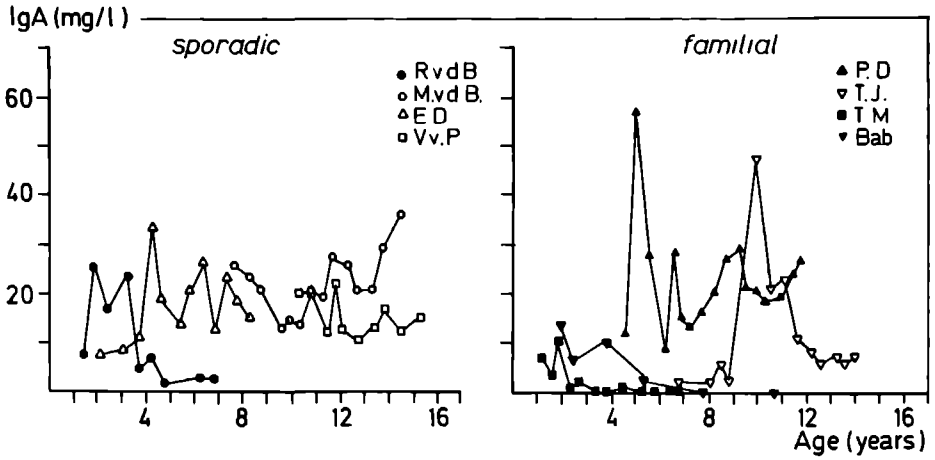


Figure 7.1: Longitudinal course of serum IgA levels in 8 children with fluctuating serum IgA levels.

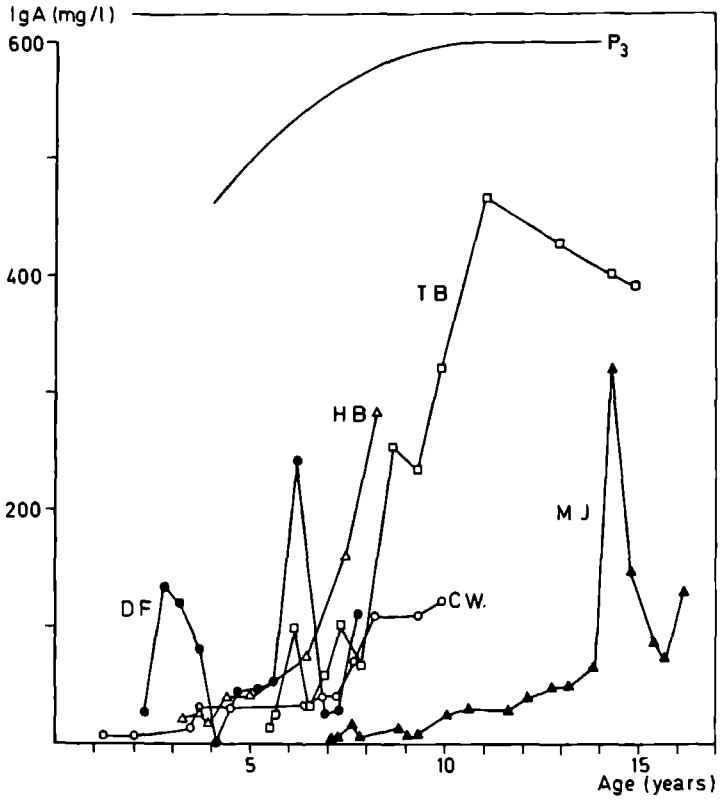


Figure 7.2: *Longitudinal course of serum IgA levels in 5 children who developed serum IgA levels above 50 mg/l during the follow-up period (Patients CW, HB and TB are "sporadic", DF and MJ are "familial" cases). The drawn line represents the P_3 percentile for serum IgA levels of healthy Dutch school children obtained in an earlier study (15).*

Longitudinal IgD levels were below 1 IU/ml in 15 children, and 12 of them had serum IgA levels below 2 mg/l. IgD levels were above 1 IU/ml in 13 children, and 10 of them had serum IgA levels above 2 mg/l.
The relationship observed between longitudinal serum IgA and IgD levels was statistically significant (p=0.007, Fisher test) in the "sporadic" group, but not in the "familial" group (Table 7.1).
Three patients developed undetectable IgD levels during follow-up. IgA levels in one of them were always below 2 mg/l. The other two patients initially developed IgA levels above 50 mg/l. Later on, coinciding with a drop in IgD levels to undetectable values, maturation of their IgA system seemed hampered (CW and DF, Figure 7.2). One of the "familial" patients (PD, Figure 7.1) demonstrated IgD deficiency during the entire follow-up period while IgA levels remained around 20 mg/l. In 7 children with variable IgA levels (above 2 mg/l) and in 3 children with serum IgA levels above 50 mg/l, IgD levels were above 1 IU/ml (Table 7.1).

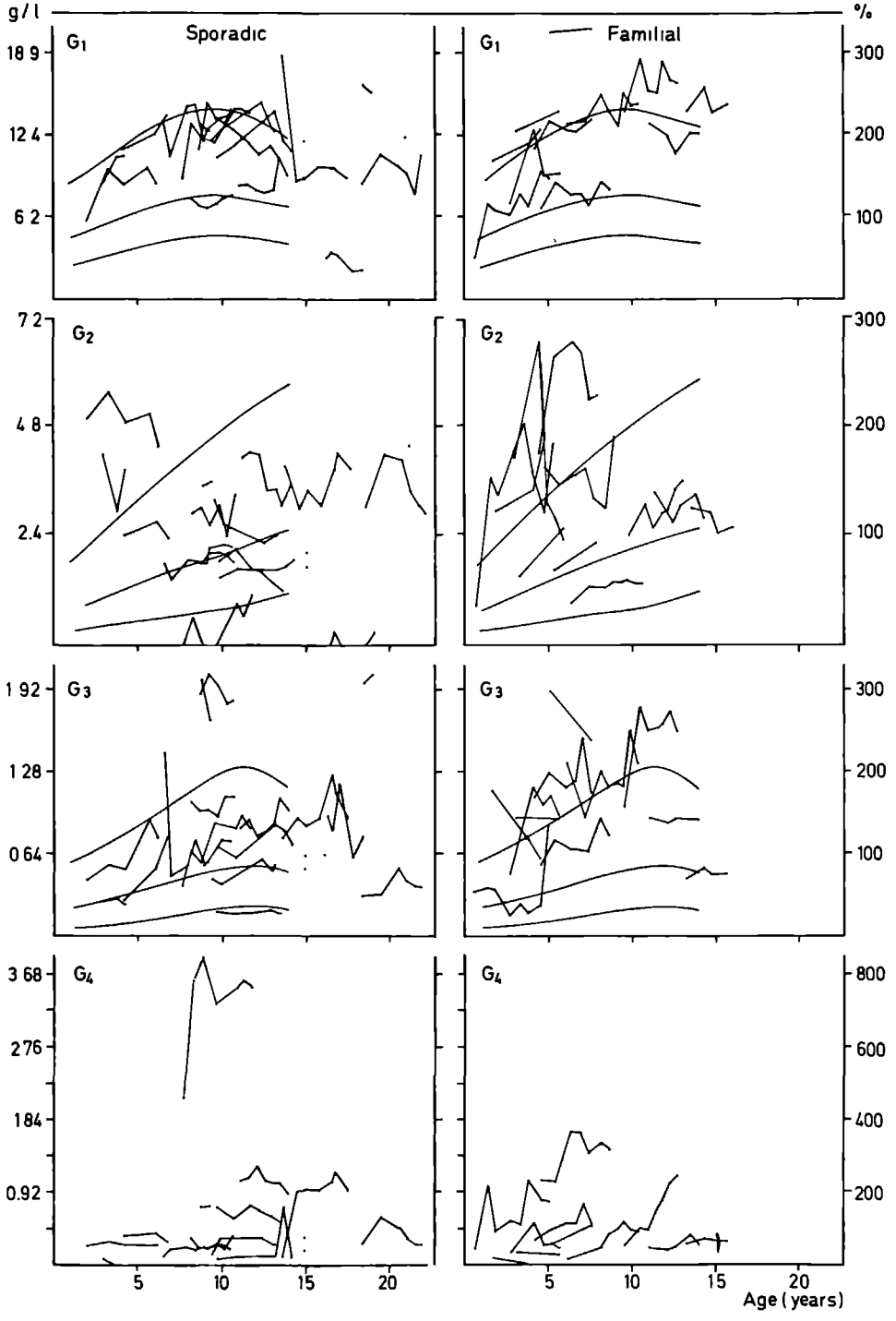
Table 7.1: Longitudinal serum IgA and IgD levels in 28 children with selective IgA deficiency (8 children were excluded because of insufficient data for IgD).

		IgD		
		<1 IU/ml	>1 IU/ml	
	Sporadic (n=17)			
IgA	<2 mg/l	9	1	p = 0.007 (Fisher test)
	>2 mg/l	1	6	
	Familial (n=11)			
IgA	<2 mg/l	3	2	not significant
	>2 mg/l	2	4	

IgE levels below 5 IU/ml were found in 10 children (8 "sporadic" and 2 "familial"). In 5 of the "sporadic" patients serum IgE became below 5 IU/ml during follow-up. Eleven children (5 "sporadic" and 6 "familial") demonstrated high levels of IgE. There was no consistent association between height of serum IgE and atopic complaints. Three "sporadic" patients had atopic complaints despite IgE levels below 5 IU/ml, and in two of them RAST tests for specific IgE (Phadiatop, Pharmacia Diagnostics BV, Woerden, The Netherlands) were positive. Six out of the 11 children with high IgE levels showed positive RAST tests for specific IgE, and 4 of them had atopic complaints.

Serum IgG levels were elevated significantly in both "sporadic" and "familial" patients, with already elevated levels at younger ages (13). Longitudinal IgM levels were within or just above the normal range. Longitudinal IgG subclass levels were studied in 34 patients (Figure 7.3). The increase in serum IgG levels was not found to be due to elevated concentrations of a specific IgG subclass. Particularly in the "familial" group, several patients demonstrated already (very) high IgG_2 levels at young ages (Figure 7.3). Concomitant IgG_2-IgG_4 deficiency was found in two boys both belonging to the "sporadic" group. One was a mentally retarded child with ring chromosome 22 but no other complaints. The other boy had considerably lowered serum IgG levels. During follow-up his serum IgG and IgM levels further declined, and he was considered to be a case of late-onset hypogammaglobulinaemia (13). In one patient IgG_2 levels were low at first, but increased during follow-up (Figure 7.3). His IgG_4 levels were very high (above 3 g/l). Undetectable IgG_4 levels alone were noted in 5 children (4 "sporadic" and 1 "familial").

Figure 7.3: *Longitudinal course of IgG subclass levels in 32 children with selective IgA deficiency. Age specific reference values (3rd percentile curve, expected value determined using regression analysis, and 97th percentile curve) for IgG_1, IgG_2 and IgG_3 are according to Plebani et al. (17). The right axis refers to the percentage of the reference serum.*


g/l
%
Sporadic
Familial
G1
G2
G3
G4
18 9
12 4
6 2
7 2
4 8
2.4
1 92
1 28
0 64
3 68
2 76
1 84
0.92
300
200
100
800
600
400
5
10
15
20
Age (years)

7.4.2 IgA in saliva

Secretory IgA was measured once or twice in saliva of 32 children. If serum IgA levels were below 2 mg/l, saliva IgA levels were also below 2 mg/l in all patients. Saliva IgA measurements in children with longitudinal serum IgA levels above 2 mg/l are shown in Table 7.2. In the children with serum IgA levels increasing above 50 mg/l, IgA was also detectable in saliva (Figure 7.2, Table 7.2).

Table 7.2: Saliva IgA in children with serum IgA levels above 2 mg/l.

	IgA					
	1st Sample			2nd Sample		
Patient	age (yrs)	serum (mg/l)	saliva (mg/l)	age (yrs)	serum (mg/l)	saliva (mg/l)
sporadic						
R.vdB.	1	14	<2	6	2.3	<2
E.D.	2	8	8	8	19	32
M.vdB.	8	20	<2	11	19	11.1
V.P.	10	20	<2			
C.W.	4	31	<2	9	112	39
H.B.	4	23	6.6			
T.B.	6	32	<2	14	380	139
familial*						
T.M.	1	7.6	<2			
P.D.	4	11.7	4.2	9	21.2	41.1
T.J.	7	2.2	<2	13	6.9	3.5
D.F.	5	45.1	3.7			
M.J.	7	7.0	<2	15	75	86

* = patient Ba.B. (Figure 7.1) was not tested for saliva IgA.

7.5 DISCUSSION

In patients without anti-IgA antibodies serum IgA was always detectable if measured by a sensitive radioimmunoassay, but most children demonstrated persistence of very low levels (<2 mg/l). Although still within the range set for definition of IgA deficiency (<50 mg/l), in some patients considerable intra-individual variance was noted. These findings are in agreement with Laschinger et al. who, in order to establish an IgA deficient blood donor panel, still found considerable fluctuations in serum IgA concentrations even below a level of 0.5 mg IgA/l (11). The fluctuations in serum IgA levels found in some of our patients were not related with infectious episodes and there were no simultaneous fluctuations in serum IgG or IgM levels. Studies on IgA heavy chain constant region genes (situated on chromosome 14) have revealed that there are no structural deletions in individuals with selective IgA deficiency (19). The underlying cause of IgA deficiency generally is considered to be a disturbance in the regulation of B cell differentiation to IgA-secreting plasma cells (20-22). Supposing that these regulatory disturbances are not constant in all patients, this could explain why in some patients fluctuating IgA levels can be found.

Five children developed serum IgA levels above 50 mg/l and IgA was also detectable in saliva, but during the available follow-up period none of them attained the lower range of age and sex specific reference values. However, if IgA levels will continue to rise, perhaps these patients will represent cases of transient IgA deficiency as reported previously (4,12). It has to be noted that in our study maturation of the IgA system still occurred at relatively advanced ages. This is in contrast with Plebani et al. who found that IgA deficiency in children with IgA levels below 50 mg/l generally is persistent (23).

In agreement with the elevation of longitudinal IgG levels in our patients (13), longitudinal IgG subclass levels (including IgG_2) in most of our patients, suffering from only relatively mild infections, generally were found to be rather high. Elevated IgG subclass levels in selective IgA deficiency have been described by Klemola (8), but most of these patients were suffering from active diseases (juvenile chronic arthritis and coeliac disease). One of our patients developed late-onset hypogammaglobulinemia. Recently a common susceptibility gene located in the MHC class III region on chromosome 6 has been implicated for both selective IgA deficiency and common variable immunodeficiency, suggesting that these are related disorders (22,24).

In the "sporadic" group but not in the "familial" group, a statistically significant association between IgD deficiency and serum IgA levels below 2 mg/l, and between detectable serum IgD and serum IgA levels above 2 mg/l was found. Two children with serum IgA levels above 50 mg/l, who thusfar did not fully mature to normal IgA levels, developed IgD deficiency in the same period. Concomitant IgD deficiency in individuals with IgA deficiency has been reported previously (7,10). The role of IgD in the regulation of the immune response is not clear as yet. In a sequence of studies by Coico and coworkers an immuno-augmenting effect of IgD in both primary and secondary responses has been described in the murine system (25). Injection of myeloma-derived IgD caused an increase in the numbers of IgA- and IgG_2-producing spleen cells. It was shown that the immuno-augmenting effect of IgD was T cell-dependent (25). T cells with receptors for IgD (T-delta cells) involved in the humoral immune response have also been demonstrated in humans (26). The relation between longitudinal IgA and IgD levels in our patients could suggest an immunoregulatory role for IgD in the development of a mature IgA system. The difference observed in IgA and IgD levels between both groups then would be supportive for a difference in the mechanism causing IgA deficiency in "sporadic" and "familial" patients. However, another explanation for the relation observed between IgA and IgD levels to be considered would be that both are influenced by a common mechanism regulating B cell differentiation.

In conclusion, apart from the statistically significant association observed between longitudinal serum IgA and IgD levels in the "sporadic" group, there were no differences in longitudinal immunoglobulin levels between the "sporadic" and "familial" cases of selective IgA deficiency presented in this study.

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Chapter 8

DISCUSSION AND CONCLUDING REMARKS

8.1 CLINICAL MANIFESTATIONS

Clinical manifestations were studied longitudinally in the two groups of "sporadic" and "familial" cases with selective IgA deficiency. All patients had undetectable or extremely low levels of secretory IgA in saliva. The majority of the children of both groups suffered from recurrent respiratory tract infections (RRTI), especially recurrent otitis media. There were no differences between "sporadic" and "familial" cases. Infection episodes generally followed a mild course and, although RRTI were more frequently found in early childhood, also at older ages RRTI often occurred. In this way the incidence of RRTI was not merely a reflection of age, comparable to healthy children, but appeared to be really increased. Furthermore, the 12 children who were not referred because of complaints also demonstrated RRTI during follow-up. From these findings it was concluded that in both patient groups there was a clear association between selective IgA deficiency and RRTI (Chapter 2).

IgG subclass deficiencies (mainly IgG_2) are known to predispose to more frequent and severe respiratory tract infections (1). Only two children had concomitant IgG_2-IgG_4 deficiency: both were "sporadic" cases, one child was asymptomatic, the other developed late onset hypogammaglobulinemia (patients PM and PN in Chapter 2). Isolated IgG_2 deficiency was not found. Isolated IgG_4 deficiency was found in 8 (20%) IgA deficient children (4 of each group). Clinical symptoms in IgG_4-IgA deficient patients were not different from those in IgA deficient patients without concomitant IgG_4 deficiency. The percentage found for IgG_4 deficiency was similar to the percentage reported for the normal population (2). Longitudinal IgG but not IgM levels were elevated significantly in both groups, and all IgG subclasses contributed to this elevation (Chapter 7). In accordance with recent reports (3,4), in young children with selective IgA deficiency a tendency was noted for high IgG subclass levels. Whether the elevated IgG levels must be regarded as a compensatory mechanism for IgA deficiency, or merely are a reflection of frequent infections remains a difficult question. It has been reported that the immune response after oral cholera vaccination, in normal individuals predominantly of the IgA class, in symptomatic IgA deficient patients was often of the IgG class (5).

The relation was studied between the clinical manifestations observed in both patient groups and the pathogenic mechanism found responsible for defective in vitro IgA production (Chapter 4). In a small number of children diminished T helper function was found for both in

vitro IgA and IgM production. If this in vitro phenomenon forms a correct reflection of the in vivo immune status, these children could have a more severe immunodeficiency compared to others with selective IgA deficiency. Some evidence for this hypothesis could be found in the fact that the two children with more severe infections (osteomyelitis and recurrent purulent lymphadenitis) both belonged to this group (Chapter 4). Furthermore, it was found that clinical symptoms other than RRTI or atopic disease mainly were recorded in patients who also had disturbances in in vitro IgM production. The heterogeneity of clinical manifestations in selective IgA deficiency could be based on the fact that some patients tend to develop more general disturbances in the regulation of immunoglobulin synthesis and consequently have more complaints. One patient who had severe RRTI and an episode of Haemophilus influenzae meningitis developed common variable immunodeficiency (CVID) during the follow-up period (Chapter 2). In the 3 patients with gold-induced decreases in serum IgA levels no increased rate of infections was observed, although at least one of them also had a disturbance in in vitro IgM production (Chapter 6).

A difference between both groups was noted in the occurrence of atopic complaints (Chapter 2). Only in the "sporadic" group a high incidence was found, so it appeared that allergic states are more frequently encountered in "sporadic" cases of selective IgA deficiency. It is clear that a definite conclusion in this matter requires a study of two much larger groups of "sporadic" and "familial" patients. Follow-up study of the longitudinal serum IgE levels in both groups showed that there was no association between height of serum IgE levels and the occurrence of atopic complaints, nor with the rate of infections. Concomitant IgE deficiency was found in 13 children. Elevated IgE levels were found in 11 children, but only 6 of them suffered from atopic complaints (Chapter 2). Serum IgE levels in individuals with IgA deficiency apparently can be elevated independent of the occurrence of atopic disease. Research on T cell-derived cytokines has revealed that interleukin (IL)-4 is not only important for IgA B cell differentiation, but is also involved in the pathways towards production of IgG_1 and IgE (6). Perhaps in some individuals with selective IgA deficiency the defect in IgA production generates an overproduction of IL-4. This could then result in an overshoot of IgE and IgG_1 production, causing an increase in the respective serum levels.

Compared to normal individuals elevated IgM and IgG levels have been reported in secretions of patients with selective IgA deficiency (7,8), but no differences were found between symptomatic and healthy IgA deficient individuals (8). We investigated saliva IgG and IgM levels once or twice in 16 children (10 "sporadic" and 6 "familial" cases). The results were heterogeneous: saliva IgG ranged from 13 to 101 mg/l (mean 40 mg/l), and IgM from 5 to 122 mg/l (mean 33 mg/l). Interpretation was hampered by the facts that the ages at the time of the saliva collections varied from 3 to 15 years, and that six of the children belonged to the group of patients with variable serum and saliva IgA levels. It only could be concluded that the saliva IgG and IgM levels found in the patients studied in this thesis appeared to be higher than those in the control group reported by Norhagen et al. (8): median IgG 10.1 mg/l, median IgM 6.3 mg/l.

In 5 children (3 "sporadic" and 2 "familial" cases) serum IgA levels rose above 50 mg/l, and IgA was detectable in saliva (Chapter 7). None of them has yet reached the lower range of reference IgA levels. However, if serum IgA levels continue to rise they will have to be considered as cases of transient IgA deficiency. The frequency of respiratory tract infections and atopic complaints was found to decline. The follow-up of these children further demonstrated that maturation of the IgA system can still occur at relatively advanced ages. This finding is in contrast with previous reports, in which was stated that so-called severe or absolute IgA deficiency (IgA levels <50 mg/l) usually is persistent (9,10).

8.2 ANTI-IgA ANTIBODIES

The role of anti-IgA antibodies in the pathogenesis of selective IgA deficiency is not fully understood. Several studies have reported a higher frequency of mother-to-child inheritance of selective IgA deficiency than of father-to-child inheritance (11-13). It has been suggested that transplacental passage of IgG class anti-IgA antibodies could result in IgA deficiency in the infant (14-16). The capability of anti-IgA antibodies to induce the formation of IgA-specific T suppressor cells that inhibit IgA production by affecting IgA B cell development has been demonstrated in the murine system (17).

Four children from two families in which IgA deficient mothers had circulating class-specific anti-IgA were investigated. In one family 2

children were followed from birth: one of them (patient NT) was IgA deficient from birth, his younger brother (patient YT) had normal serum IgA levels in his first year of life but turned out to be IgA deficient at the age of five. In the other family immunoglobulin levels in early childhood were not available. The in vitro experiments revealed that in all four children B cell defects were indeed combined with excessive IgA-specific T suppressor function, thus strongly suggesting that IgA deficiency in the progeny was the result of maternal anti-IgA antibody-induced excessive T suppressor function that subsequently had affected IgA B cell differentiation (Chapter 5). The findings in patient YT suggested that this influence on IgA B cell differentiation apparently was not limited to the prenatal period but can still take place a long time after birth and is not caused by at that time circulating anti-IgA antibodies.

Three of the 4 children from IgA deficient mothers with circulating anti-IgA antibodies developed anti-IgA antibodies themselves up till now. All three children and also their mothers had concomitant IgG_4-IgE deficiency. Patient NT was IgA deficient from birth, but IgE levels initially were normal. At the age of three he became IgE deficient and IgA antibodies were first detected. Development of anti-IgA antibodies in this distinct group of patients apparently can occur anytime between birth and puberty, as in one of the children (patient AL) it was observed only at the age of nine. Patient YT had normal levels of serum IgG_4 and IgE up till now and anti-IgA antibodies could not be detected as yet.

During the follow-up period of the other 36 children, only in one other patient (patient MC, Chapter 2) class-specific anti-IgA antibodies could be detected. She was known with IgG_4-IgA deficiency and developed anti-IgA antibodies at the age of eighteen. At the same time she was found to have become IgE deficient.

Thus, all 6 individuals (4 children and 2 mothers) in this study with presence of circulating anti-IgA antibodies had concomitant IgG_4-IgE deficiency. Increased frequencies of anti-IgA antibodies in IgA deficient patients with concomitant IgG subclass and/or IgE deficiency have been reported, although the explanation for this association is unknown (18-20). Development of anti-IgA antibodies before puberty was only found in the children from IgA deficient mothers with anti-IgA antibodies. Perhaps periodic screening for anti-IgA antibodies in children with selective IgA deficiency until puberty should be reserved for cases with concomitant IgG subclass and/or IgE deficiency, and for IgA deficient children of IgA deficient mothers with circulating anti-IgA antibodies.

8.3 PATHOGENESIS OF SELECTIVE IgA DEFICIENCY

The major cause underlying selective IgA deficiency is thought to be defective IgA B cell differentiation and maturation. Although on rare occasions structural defects in the alpha heavy chain gene have been demonstrated, this never affected both the alpha-1 and alpha-2 gene (13). The individuals with these deletions were not totally IgA deficient, but lacked either IgA_1 or IgA_2. Our findings that two of the patients who possibly had transient IgA deficiency were "familial" cases (patients DF and MJ, Chapter 7), and that another "familial" patient (patient HB) had an identical twin brother without IgA deficiency also suggested that structural gene deletions are not likely to play an important role in selective IgA deficiency. Hammarström et al. have reported that in most individuals with selective IgA deficiency there were no structural gene deletions in the IgA heavy chain constant region genes on chromosome 14 (21). This has led in general to the conclusion that in IgA deficiency there is a disturbance in the regulation of terminal differentiation of B cells to IgA-secreting plasma cells. Up till now the exact mechanisms involved in the regulation of IgA synthesis are not clear. Regulatory genes for IgA synthesis have been suggested, but have not been precisely located as yet.

The association of selective IgA deficiency with aberrations in chromosome 18 is well-known. One of the patients also had presence of a ring-chromosome 18 (patient EvdB, Chapter 2). The role of gene products of chromosome 18 in IgA synthesis is difficult to interpret because IgA deficiency has been associated as well with deletions of the short arm (18p-), as with deletions of the long arm (18q-), and with ring-chromosome 18; possibly multiple loci are involved (13). The other patient with a chromosomal abnormality group (patient PM, Chapter 2) had a ring-chromosome 22. He belonged to a large family, and none of the family members had IgA deficiency. An association between aberrations of chromosome 22 and selective IgA deficiency has not been reported before. Both patients were mentally retarded and had physical abnormalities. Chromosomal analysis was also performed in 14 IgA deficient children without mental deficiency and showed no abnormalities (22), in accordance with a previous report in which 80 IgA deficient individuals were investigated (23). It was concluded that chromosomal analysis is only recommendable in IgA deficient individuals with mental deficiency or physical abnormalities (Chapter 2).

Associations of selective IgA deficiency with particular combinations of major histocompatibility complex alleles of all three classes (so-called MHC supratypes) have been reported (24-27). These findings suggest that genes regulating IgA B cell differentiation could be located within or near the MHC complex on chromosome 6. The regulatory disturbance in B cell differentiation can be limited to IgA synthesis only, as was the case in 14 of the patients who had true "selective" IgA deficiency. However, additional deficiencies of other immunoglobulins (IgG subclasses, IgD, IgE) are not unusual in patients with selective IgA deficiency (3,4,9,28-30), and were also found in the majority of our patients (Chapter 7). Furthermore, in families of patients with selective IgA deficiency the incidence of other immunoglobulin deficiencies, including true hypogammaglobulinemia, is increased (9,31). One of our patients developed late onset hypogammaglobulinemia, resembling the patient described by Morrell et al. (32). Perhaps selective IgA deficiency forms a more simple representative of a much broader spectrum of regulatory disturbances of immunoglobulin synthesis. In vitro studies have shown that the immunological disturbances implicated in CVID and selective IgA deficiency have a certain resemblance: intrinsic B cell defects and/or T cell regulatory disturbances. Immunogenetic studies focussing on regulatory genes for B cell differentiation have already demonstrated that susceptibility to both CVID and selective IgA deficiency appears to be determined by genes within or near the major histocompatibility complex class III gene region on chromosome 6, supporting the hypothesis that these immunodeficiency disorders are related (26,27). We cannot comment on this interesting issue as HLA typing was not performed in the present study.

In most of the patients serum IgA levels were studied during a prolonged period of time. Although the majority showed persistence of very low IgA levels (<2 mg/l), in some children, both "familial" and "sporadic", considerable fluctuations were noted (Chapter 7). There was no relation with infection episodes, and there were no simultaneous fluctuations in other immunoglobulins. Perhaps these fluctuations reflect an imbalanced regulation of IgA synthesis.

The capacity for in vitro IgA production was investigated in 26 children (in 14 "sporadic" and 8 "familial" cases in Chapter 4; and in 4 "familial" cases in connection with anti-IgA antibodies in Chapter 5). PBMC of all children but one (a child who probably had transient IgA deficiency) demonstrated defective in vitro IgA production compared to healthy controls. In an early report Waldmann and coworkers described

a secretory blockade in IgA plasma cells (14). We also have investigated this phenomenon, but none of the patients showed evidence for a secretory blockade of IgA.

The molecular and cellular interactions that regulate the isotype-specific differentiation of B cells to IgA production and secretion are profoundly influenced by T cells and T cell-derived factors (cytokines) at various stages (33). T cell abnormalities have been reported in patients with selective IgA deficiency (14,34-36). Recent developments in cytokine research have allowed the possibility to study the role of interleukins in immunodeficiency disorders. IL-5 mRNA producing cells have been investigated in individuals with selective IgA deficiency or CVID, and revealed no abnormalities compared to healthy controls (37). In patients with CVID addition of IL-2, IL-4 and IL-6 to cell cultures had only minor effects on immunoglobulin synthesis (38). However, it has to be considered that the lymphocyte populations used for in vitro cultures possibly do not contain the precursor population that is dependent on certain cytokines for differentiation. Defects in the receptors for interleukins in the relevant target cell populations have also been suggested (37).

Our in vitro culture experiments emphasized on the cellular basis of selective IgA deficiency. Three pathogenic mechanisms could be distinguished (Chapter 4): pure defective IgA B cell function, defective IgA B cell function with IgA-specific excessive T suppressor function, and defective IgA B cell function with diminished T helper function (which was not always IgA-specific). There were no differences in pathogenic mechanisms between "sporadic" and "familial" cases. Defective in vitro IgA B cell function was found in most of the cases, but in 12 out of 22 patients additional T cell disturbances could be established. This finding agrees well with the current opinion that IgA B cell differentiation is influenced at various stages by T cells and T cell-derived factors. Pure T cell abnormalities were not found in the present study. The significance of the additional T cell abnormalities remained unclear. The question remained whether T cell abnormalities (found in connection with defective IgA B cell function) represented a primary cause or became manifest later on in life. A primary role could not be substantiated as in the "familial" cases, although limited in number, a consistent pattern of T cell disturbances was found in only one of four families (Chapter 4). The excessive T suppressor function found without exception in the children from mothers with anti-IgA antibodies was thought to be significant in the pathogenesis of IgA deficiency in this

distinct group (Chapter 5). Additional excessive T suppressor function was also found in another three patients (Chapter 4), but was neither related to anti-IgA antibodies nor to clinical manifestations.

The function of IgD in the human immune system is not exactly known. The possibility of an immunoregulatory role of IgD deserves attention. IgD deficiency is often found in individuals with selective IgA deficiency (9,28,30). In the early stages of B cell development IgD is expressed on the surface of B cells and disappears afterwards in the process of isotype-switching (Chapter 1). Its role in the isotype-switch is unknown. An immuno-augmenting effect of IgD on IgA and IgG_2 immune responses mediated by T cells with receptors for IgD (T-delta cells) has been described (39). In our patients in the "sporadic" group a significant association was found between longitudinal serum IgD and IgA levels. IgD deficiency was associated with serum IgA levels <2 mg/l, and if IgD was detectable in serum in most patients IgA levels were >2 mg/l (Chapter 7). This could suggest a regulatory role for IgD in IgA synthesis. Another possibility could be that IgD and IgA differentiation share certain regulatory mechanisms in common.

Further knowledge on the factors involved in the regulation of the process of B cell differentiation towards immunoglobulin-producing plasmacells in humans needs to be obtained. The results can be used in future experiments investigating the pathogenesis of humoral immunodeficiencies including selective IgA deficiency. The role of interleukins can be further investigated by measurement of in vitro interleukin production in cell cultures, and by evaluating the effects of addition of recombinant interleukins to cell cultures on immunoglobulin synthesis. Addition of exogeneous IgD to cell cultures of IgA deficient individuals, with or without concomitant IgD deficiency, could elucidate the immunoregulatory role of IgD assumed in Chapter 7.

8.4 GENERAL CONCLUSION

The aim of this thesis was to see whether the well-known heterogeneity of patients with selective IgA deficiency could be lessened by dividing our well-documented patients into a "sporadic" and a "familial" group. Regarding the clinical follow-up data, the longitudinal immunoglobulin levels and the in vitro experiments performed to elucidate the pathogenic mechanism, no differences were found between the two groups except the more frequent observation of atopic complaints and the relation that appeared to exist between longitudinal serum IgA and IgD levels in the "sporadic" group. So the heterogeneity could not be attributed to differences between "sporadic" and "familial" patients. However, we and others have found evidence that selective IgA deficiency and CVID appear to be related disorders. The heterogeneity of selective IgA deficiency could then be explained by the assumption that some patients with selective IgA deficiency tend to shift towards development of CVID and have more and more severe complaints (for instance more severe infections or autoimmunity), while others who remain relatively asymptomatic do not.

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SUMMARY

Selective IgA deficiency was first described some 30 years ago and is considered to be the most frequent primary immunodeficiency. Many reports have been published describing various clinical and immunological aspects of this immunodeficiency state. It is concluded in almost all studies that individuals with selective IgA deficiency form a heterogeneous population.

The aim of this thesis is an effort to lessen this heterogeneity by studying a well-defined and longitudinally documented population of children and adolescents with selective IgA deficiency, dividing them into two groups of "sporadic" and "familial" cases.

Chapter 1 describes the structure and function of IgA, followed by a brief review of the main clinical and immunological aspects of selective IgA deficiency. The current knowledge on the process of B cell differentiation towards IgA secreting plasma cells and the literature upon the pathogenesis of selective IgA deficiency is summarized.

Chapter 2 describes longitudinally the clinical manifestations in 25 "sporadic" and 15 "familial" patients with selective IgA deficiency. There were no obvious differences between "sporadic" and "familial" cases. The majority of both groups had frequent respiratory tract infections, especially recurrent otitis media. Atopic complaints were also frequently found and occurred more often in the "sporadic" group. The frequency of atopic complaints was not well related with the height of serum IgE levels. Chromosomal abnormalities (ring 18 and ring 22) were only found in the two IgA deficient children who also were mentally retarded. Concomitant IgG subclass deficiencies were not frequent in the two groups. The longitudinal serum IgG levels were elevated significantly in both groups, probably reflecting the increased rate of infections. Anti-IgA antibodies were found in 4 patients, 3 of them had an IgA deficient mother with circulating class-specific anti-IgA antibodies. All 4 patients had concomitant IgG_4-IgE deficiency.

Chapter 3 introduces the pokeweed mitogen-driven cell culture system for in vitro immunoglobulin production used in this thesis to investigate the cellular basis of selective IgA deficiency. Statistical comparisons of the amounts of in vitro immunoglobulin production in cocultures of

counterpart non-T and T cell fractions of patients and healthy controls enabled the distinction of defective B cell function, excessive T suppressor function and diminished T helper function. The methods and statistical calculations are described.

Chapter 4 describes the results of in vitro immunoglobulin production in 22 children with selective IgA deficiency (14 "sporadic" and 8 "familial") in relation to the clinical manifestations. All patients but one had defective in vitro IgA production. In 4 children also in vitro IgM production was diminished. The coculture studies revealed that all but one had a B cell defect for in vitro IgA production. So in this study it was found that the pathogenic base of selective IgA deficiency at a cellular level was defective IgA B cell function. In 12 patients additional T regulatory disturbances were found. Three had IgA-specific excessive T suppressor function. Diminished T helper function was found in 4 patients for in vitro IgA production, while 5 patients had diminished T helper function for both in vitro IgA and IgM production. There were no obvious differences between "sporadic" and "familial" cases. The importance of the role of additional T cel regulatory disturbances in the pathogenesis of selective IgA deficiency is not clear. There were no differences in clinical manifestations between patients with a pure B cell defect and patients with a B cell defect and additional IgA-specific T cell regulatory disturbances. It was noted that most of the patients with more severe infections or with complaints other than recurrent respiratory tract infections or atopy also had disturbances in in vitro IgM production. Perhaps these patients have a more serious immunodeficiency shifting towards the development of common variable immunodeficiency. This hypothesis could be an explanation for the heterogeneity observed in patients with selective IgA deficiency.

Chapter 5 describes the results of the in vitro immunoglobulin production experiments in 4 "familial" children with selective IgA deficiency. They belonged to two families in which the mothers were IgA deficient and had circulating class-specific anti-IgA antibodies. In one of the families transplacental passage of maternal anti-IgA has been demonstrated. It has been suggested in the literature that in some patients with selective IgA deficiency the maturation of the IgA system is influenced in the perinatal period by maternal anti-IgA antibodies, but the mechanism remains unclear. The coculture experiments in our patients showed that all four children had an IgA B cell defect with

additional IgA-specific excessive T suppressor function. We suggest that in these children transplacental anti-IgA has generated IgA-specific T suppression that finally affected IgA B cell function. This latter process is not limited to the perinatal period as one of the children became IgA deficient after the age of one year. Three of the children developed anti-IgA antibodies at various ages but before puberty.

Chapter 6 describes 3 patients with juvenile chronic arthritis who developed a significant fall in serum IgA levels after treatment with aurothioglucose. One of them developed persistent "true" selective IgA deficiency and is still treated with gold. In the second patient serum IgA levels remained below the normal range after cessation of gold therapy. In the third patient serum IgA levels returned to pretreatment values. None of the patients had toxic side-effects and the rate of infections was not increased. In vitro immunoglobulin production experiments showed that all three had diminished IgA B cell function. At least one of the two patients who were still on gold therapy at the time the experiments were performed had an also diminished IgM B cell function, although serum IgM levels were in the normal range in both.
The conclusion of the in vitro experiments was that aurothioglucose can interfere with in vitro B cell function. Sometimes this apparently can cause a significant fall in serum immunoglobulin levels, especially of IgA. In contrast with most patients with primary selective IgA deficiency this caused no symptoms in our 3 patients.

Chapter 7 describes the longitudinal immunoglobulin levels in 36 children with selective IgA deficiency (25 "sporadic" and 11 "familial").
In the majority serum and saliva IgA levels remained below 2 mg/l. Considerable variance in serum IgA levels was noted in 8 children, but they all remained IgA deficient. Five children developed serum IgA levels above 50 mg/l at various ages, and IgA was detectable in saliva. They could represent cases of transient IgA deficiency, but none of them has reached the lower range of the normal values as yet. Most of the children demonstrated rather high IgG subclass levels, also at younger ages. There were no obvious differences in immunoglobulin levels between "sporadic" and "familial" cases except an association between IgD deficiency and serum IgA levels below 2 mg/l, and between serum levels of IgD above 1 IU/ml and of IgA above 2 mg/l, that was found to be significant in the "sporadic" group, but not in the "familial" group.

This could suggest an immunoregulatory role of IgD in the process of IgA B cell differentiation.

Chapter 8 finally provides a general discussion of the clinical and immunological findings in the two groups of patients, followed by some directions for future research.

It was concluded that there were no obvious differences between "sporadic" and "familial" patients. Anti-IgA antibodies could be demonstrated in 6 patients (4 children and 2 mothers) and they all had concomitant IgG_4-IgE deficiency. Development of anti-IgA antibodies before puberty was only found in the children of IgA deficient mothers with anti-IgA antibodies. Considering the findings in this thesis, selective IgA deficiency is based on (regulatory) disturbances in B cell differentiation. In some patients this is restricted to IgA, but others have more general disturbances that can concern other immunoglobulins. The assumption that selective IgA deficiency forms one side of a broader spectrum of disturbances in immunoglobulin synthesis with on the other side common variable immunodeficiency, would be a reasonable explanation for the heterogeneity observed in patients with selective IgA deficiency.

SAMENVATTING

Selectieve IgA deficiëntie is 30 jaar geleden voor het eerst beschreven en wordt beschouwd als de meest frequent voorkomende primaire immuundeficiëntie. Er zijn vele publicaties verschenen over allerlei klinische en immunologische aspecten van deze immuundeficiëntie. Een belangrijke conclusie uit bijna alle studies is dat individuen met selectieve IgA deficiëntie een heterogene populatie vormen.

Het doel van dit proefschrift is een poging om deze heterogeniteit te verminderen door een goed gedefinieerde en longitudinaal vervolgde populatie van kinderen en adolescenten met selectieve IgA deficiëntie te bestuderen, onderverdeeld in twee groepen: "sporadische" en "familiaire" patienten.

Hoofdstuk 1 beschrijft de structuur en functie van IgA, gevolgd door een kort overzicht van de belangrijkste klinische en immunologische aspecten van selectieve IgA deficiëntie. Een samenvatting van de huidige kennis over het proces van B cel differentiatie tot IgA secreterende plasmacellen en de literatuur over de mogelijke pathogenese van selectieve IgA deficiëntie besluit het hoofdstuk.

Hoofdstuk 2 beschrijft longitudinaal de klinische verschijnselen bij 25 "sporadische" en 15 "familiaire" patienten met selectieve IgA deficiëntie. Er werden geen duidelijke verschillen gevonden tussen "sporadische" en "familiaire" patienten. In beide groepen had de meerderheid van de patienten recidiverende luchtweginfecties, met name recidiverende otitis media. Atopische klachten werden ook frequent gevonden, en kwamen vaker voor in de "sporadische" groep. Er bestond geen duidelijke correlatie tussen de hoogte van de serum IgE concentraties en het voorkomen van atopische klachten. Chromosomale afwijkingen (ring 18 en ring 22) werden alleen gevonden bij kinderen met selectieve IgA deficiëntie die tevens mentaal geretardeerd waren. Het tegelijk voorkomen van IgG subklasse deficiënties werd in beide groepen slechts bij enkele patienten aangetoond. In beide groepen bleken de longitudinale serum IgG concentraties significant verhoogd te zijn, waarschijnlijk als gevolg van het verhoogde aantal infecties. Anti-IgA antistoffen waren aantoonbaar in 4 patienten, 3 van hen hadden een moeder met IgA deficiëntie en circulerende klasse-specifieke anti-IgA antistoffen. Alle 4 patienten hadden tevens een IgG_4-IgE deficiëntie.

Hoofdstuk 3 beschrijft het pokeweed mitogeen-gestimuleerde celkweeksysteem voor in vitro immunoglobuline productie dat in dit proefschrift gebruikt werd om de cellulaire basis van selectieve IgA deficiëntie te bestuderen. Door de in vitro immunoglobuline producties in gemengde celkweken van non-T en T cel fracties van patienten en gezonde controles statistisch met elkaar te vergelijken was het mogelijk om een defect in B cel functie, excessieve T suppressor functie en verminderde T helper cel functie te onderscheiden. De gebruikte methoden en de statistische berekeningen worden beschreven.

Hoofdstuk 4 beschrijft de resultaten van in vitro immunoglobuline productie in 22 kinderen met selectieve IgA deficiëntie (14 "sporadische" en 8 "familiaire") in relatie met de klinische verschijnselen. Alle patienten op één na hadden een defect voor in vitro IgA productie. In de gemengde celkweken bleek dat alle patienten op één na een B cel defect hadden voor in vitro IgA productie. Als belangrijkste oorzaak voor selectieve IgA deficiëntie op cellulair niveau werd in deze studie dus een IgA B cel defect gevonden. In 12 patienten werden tevens T cel regulatie- stoornissen gevonden. Drie hadden IgA-specifieke excessieve T suppressor functie. Een verminderde T helper aktiviteit was in 4 patienten aantoonbaar voor in vitro IgA productie, terwijl 5 patienten een verminderde T helperfunctie toonden voor zowel in vitro IgA als IgM productie. Er werden geen duidelijke verschillen waargenomen tussen "sporadische" en "familiaire" patienten. Het is niet duidelijk welke betekenis aan de additionele T cel regulatiestoornissen moet worden toegekend in de pathogenese van selectieve IgA deficiëntie. Er werd geen verschil gevonden in klinische verschijnselen tussen patienten met uitsluitend een B cel defect en patienten met een B cel defect en tevens IgA-specifieke T cel regulatiestoornissen. Het viel op dat bijna alle patienten met infecties van ernstiger aard, dan wel klachten anders dan recidiverende luchtweginfecties of atopie, tevens een afwijkende in vitro IgM productie hadden. Misschien hebben deze patienten een uitgebreidere immuundeficiëntie in die zin dat zij in de richting gaan van een common variable immunodeficiency. Deze hypothese zou een verklaring kunnen vormen voor de heterogeniteit die waargenomen wordt in patienten met selectieve IgA deficiëntie.

Hoofdstuk 5 beschrijft de resultaten van de in vitro immunoglobuline productie experimenten in 4 "familiaire" kinderen met selectieve IgA deficiëntie. Zij behoren tot twee families waarin de moeders IgA deficiënt

zijn en circulerende anti-IgA antistoffen hebben. In één van de families is transplacentaire passage van maternale anti-IgA antistoffen aangetoond. In de literatuur is gesuggereerd dat in sommige patienten met selectieve IgA deficiëntie het IgA systeem in de perinatale periode beïnvloed is door maternale anti-IgA antistoffen, hoewel het mechanisme onduidelijk bleef. De gemengde celkweken in onze patienten lieten zien dat alle vier de kinderen een IgA B cel defect hadden gecombineerd met IgA-specifieke excessieve T suppressor functie. Wij zijn van mening dat de passage van anti-IgA antistoffen in de kinderen excessieve T suppressor aktiviteit heeft geïnduceerd die uiteindelijk de IgA B cel functie aantast. Dit laatste is niet beperkt tot de perinatale periode daar één van de kinderen pas IgA deficiënt werd na de leeftijd van een jaar. Drie van de kinderen ontwikkelden anti-IgA antistoffen op verschillende leeftijden doch vóór de puberteit.

Hoofdstuk 6 beschrijft 3 patienten met juveniele chronische arthritis bij wie een duidelijke daling ontstond van de serum IgA concentraties na behandeling met aurothioglucose. Eén van hen ontwikkelde een persisterende "echte" selectieve IgA deficiëntie en wordt nog steeds behandeld met goud. In de tweede patient bleven de serum IgA concentraties ver onder de normaalwaarden na staken van de goudinjecties. In de derde patient herstelden de serum IgA concentraties zich tot de waarden gemeten voor de start van de behandeling. Toxische bijwerkingen van goud werden niet waargenomen, en er was geen toename van infecties. In vitro immunoglobuline productie experimenten lieten zien dat alle drie patienten een verminderde IgA B cel functie hadden. Tenminste één van de twee patienten die nog met goud behandeld werden op het tijdstip van de celkweken had ook een verminderde IgM B celfunctie, hoewel in beiden de serum IgM concentraties normaal waren. Uit de in vitro experimenten kon geconcludeerd worden dat aurothioglucose kan interfereren met in vitro B cel functie. In sommige patienten kan hierdoor een daling in immunoglobuline concentraties ontstaan, met name van IgA. In tegenstelling tot de meeste patienten met primaire selectieve IgA deficiëntie veroorzaakte dit in onze 3 patienten geen symptomen.

Hoofdstuk 7 toont de longitudinale immunoglobuline concentraties in 36 kinderen met selectieve IgA deficiëntie (25 "sporadisch" en 11 "familiair"). In de meeste kinderen bleven de serum en speeksel IgA concentraties onder de 2 mg/l. In 8 kinderen werden forse schommelingen

waargenomen van de serum IgA concentraties, maar allen bleven IgA deficiënt. Vijf kinderen ontwikkelden op verschillende leeftijden serum IgA concentraties boven 50 mg/l en IgA was aantoonbaar in speeksel. Zij zouden beschouwd kunnen worden als transiënt IgA deficiënt, echter geen van hen heeft tot nu toe de ondergrens van de normaalwaarden voor IgA bereikt. De serum IgG subklasse concentraties waren, zelfs al op jonge leeftijd, bij de meeste kinderen vrij hoog. Er werden geen duidelijke verschillen in immunoglobuline concentraties waargenomen tussen de twee groepen behalve een associatie tussen enerzijds IgD deficiëntie en serum IgA onder 2 mg/l en anderzijds tussen IgD boven 1 IU/ml en serum IgA boven 2 mg/l, hetgeen statistisch significant was in de "sporadische" groep maar niet in de "familiaire" groep. Dit zou kunnen wijzen op een immunoregulatoire rol van IgD in het proces van IgA B cel differentiatie.

Hoofdstuk 8 bestaat tenslotte uit een algemene bespreking van de klinische en immunologische bevindingen in de twee groepen patienten met selectieve IgA deficiëntie, gevolgd door enkele suggesties voor toekomstig onderzoek.

Concluderend bleek dat er nauwelijks verschillen aangetoond kunnen worden tussen "sporadische" en "familiaire" patienten. De heterogeniteit van selectieve IgA deficiëntie kan aldus niet verklaard worden. Anti-IgA antistoffen werden gevonden bij 6 patienten (4 kinderen en 2 moeders) en allen hadden tevens een IgG_4-IgE deficiëntie. Ontwikkeling van anti-IgA antistoffen vóór de puberteit werd alleen gevonden bij de kinderen van moeders met IgA deficiëntie en anti-IgA antistoffen. Op grond van de bevindingen in dit proefschrift is selectieve IgA deficiëntie gebaseerd op (regulatoire) stoornissen in B cel differentiatie. In sommige patienten is dit beperkt tot IgA, in anderen bestaan meer uitgebreide stoornissen die ook andere immunoglobulines kunnen betreffen. De veronderstelling dat selectieve IgA deficiëntie één zijde vormt van een breder spectrum van stoornissen in immunoglobuline synthese met aan de andere kant common variable immunodeficiency, zou een redelijke verklaring kunnen zijn voor de heterogeniteit van patienten met selectieve IgA deficiëntie.

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Dit proefschrift is tot stand gekomen dankzij de hulp van velen.
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CURRICULUM VITAE

De auteur van dit proefschrift werd op 12 maart 1958 geboren te Tilburg. Hij behaalde in 1976 het Atheneum B diploma aan het Pauluslyceum te Tilburg. Vanaf 1976 studeerde hij Geneeskunde aan de Katholieke Universiteit te Nijmegen, en behaalde op 8 juli 1983 het artsexamen. Van september 1983 tot november 1984 was hij werkzaam als onderdeelsarts ter vervulling van de militaire dienstplicht.

De opleiding tot kinderarts werd aangevangen in november 1984 in het Radboudziekenhuis te Nijmegen (Opleider: Prof.Dr. G.B.A. Stoelinga). De opleiding werd tijdelijk onderbroken van november 1985 tot mei 1986. In die periode werd het hem dankzij een subsidie van de Facultaire arts-assistentenpool mogelijk gemaakt om onder leiding van Dr. C.M.R. Weemaes de basis te leggen van het onderzoek dat uiteindelijk geleid heeft tot dit proefschrift. Hij was toen werkzaam op het Cel en Weefselkweek laboratorium (Hoofd: Dr. J.A.J.M. Bakkeren) van het Laboratorium Kindergeneeskunde en Chirurgie (voormalig Hoofd: Dr. P.J.J. van Munster).

De opleiding werd in mei 1986 voortgezet en hij werd op 1 april 1989 ingeschreven als kinderarts in het specialistenregister. Sindsdien is hij werkzaam op de afdeling Kindergeneeskunde (Hoofd: Prof.Dr. H.K.A. Visser) van het Sophia Kinderziekenhuis te Rotterdam.

Hij is gehuwd met Elly Reijgersberg en vader van Paul en Frank.

Dit proefschrift is geschreven op een XT-PC met behulp van WP 5.0. Het gebruikte lettertype is Swiss Roman 12pt voor de tekst, Swiss Roman 10pt voor de tabellen en de referenties, en Swiss Italic 10pt voor de figuren. De regelafstand is 1,15. De marges zijn 2,54 cm rondom. Het is geprint op een laserprinter en daarna door de drukker 80% verkleind.

Haveka B.V. Alblasserdam

STELLINGEN

behorende bij het proefschrift

SELECTIVE IgA DEFICIENCY IN CHILDHOOD

Clinical manifestations and in vitro experiments

In het openbaar te verdedigen
op woensdag 25 maart 1992
des namiddags te 1.30 uur

door

PETER C.J. DE LAAT

1. Selectieve IgA deficiëntie is op de kinderleeftijd vooral geassocieerd met recidiverende luchtweginfecties, in het bijzonder otitis media.

(dit proefschrift)

2. Atopische klachten worden frequent aangetroffen bij patienten met selectieve IgA deficiëntie maar tonen dan vaak geen goede correlatie met de hoogte van de serum IgE concentraties.

(dit proefschrift)

3. Selectieve IgA deficiëntie wordt veroorzaakt door een regulatiestoornis in het proces van B cel differentiatie tot rijpe IgA secreterende plasmacel.

(dit proefschrift)

4. Het feit dat een aantal patienten immunologisch gesitueerd moet worden tussen selectieve IgA deficiëntie en common variable immunodeficiency kan de heterogeniteit van patienten met selectieve IgA deficiëntie verklaren.

(dit proefschrift)

5. Transplacentaire passage van anti-IgA antistoffen van moeder naar kind kan de oorzaak zijn van IgA deficiëntie bij het kind. Bij deze kinderen ontstaan dan anti-IgA antistoffen vóór de puberteit.

(dit proefschrift)

6. Medium-chain acylCoA-dehydrogenasedeficiëntie (MCAD) is in N-W Europa één van de meest voorkomende "inborn errors" van de beta-oxidatie van vetzuren en kan verantwoordelijk zijn voor SIDS en Reye-like syndrome op de vroege kinderleeftijd. In families met onbegrepen onverwachte dood dient daarom MCAD bij siblings van SIDS te worden uitgesloten.

(Blakemore et al. Lancet 1991;337:298-9.)

7. De RAAS-antagonerende, vochtmobiliserende en longvaatverwijdende eigenschappen van het Atrium-Natriuretische Peptide (ANP), en de positieve correlatie tussen verhoogde plasma ANP concentraties en de mate van ductusflow bij de pasgeborene, doen een belangrijke rol van dit hormoon vermoeden bij de aanpassing van de foetale circulatie aan het extra-uteriene bestaan.

(Semmekrot et al. Biol Neonate (in press))

8. Bij de combinatie van neurologische uitvalsverschijnselen en microcytaire anaemie dient ook in Nederland nog steeds gedacht te worden aan een loodintoxicatie.

9. De toenemende subspecialisatie in de kindergeneeskunde in de academische kinderklinieken houdt het gevaar in dat de "integrale" kindergeneeskundige zorg tekort gaat schieten. Dit kan slechts voorkomen worden door een duidelijke plaats te geven aan academische kinderartsen die functioneren als generalist.

10. De voorkeur van de gemiddelde Nederlander voor het kiezen van een "middenweg" weerspiegelt zich ook maar al te vaak in het weggedrag op autowegen met meer dan twee rijstroken.

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